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OLESIA IGNATENKO

ASTROCYTE CONTRIBUTION TO THE PATHOGENESIS OF MITOCHONDRIAL DYSFUNCTION

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ACADEMIC DISSERTATION

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Cover graphics: Astrocytes in the style of Suprematist Composition (Kazimir Malevich, 1916). The suprematism is focused on translation of phenomena through basic geometric forms and colours; this thesis takes a reductionist approach to the complexity of the central nervous system. Mouse brain, immunostaining against glial fibrillary acidic protein, counterstain with nuclear DNA. Neural Style Transfer by Rustem Kasymov.

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'People who do not want to think about death
and the difficulty of human existence,
can sit in their room all day, doing geometry'.

Blaise Pascal, interpreted by Hubert Dreyfus

ABSTRACT

Mitochondria are organelles critical for cellular energy metabolism and homeostasis. Pathogenic DNA variants that disrupt organelle function manifest as a heterogeneous group of diseases. These include severe brain encephalopathies that lack curative treatments, leading to early childhood lethality. Typical findings in brain samples of patients with mitochondrial encephalopathies include neuronal degeneration and histopathological changes of non-neuronal cells, referred to as reactive gliosis. The severe manifestations of mitochondrial encephalopathies have thus far been explained by the vulnerability of neurons to mitochondrial dysfunction, while reactive gliosis is considered a secondary response to the neuronal pathology.

In my thesis research, I used genetically modified mouse models to investigate the cell-specific contribution to the pathogenesis of mitochondrial dysfunction in the central nervous system. Using Cre-Lox recombination, the gene encoding the mitochondrial DNA helicase Twinkle was conditionally disrupted in postnatal astrocytes or neurons. In neurons, we observed the well-established vulnerability to mitochondrial dysfunction. Whereas in astrocytes, our data show reactive astrogliosis as a cell-autonomous response to mitochondrial dysfunction. Furthermore, the formation of microscopic vacuoles in the brain characteristic of spongiotic encephalopathies was only observed upon mitochondrial dysfunction in astrocytes. The same pathology also occurred upon disruption in astrocytes of the gene *Cox10*, encoding a factor essential for the assembly and function of the oxidative phosphorylation enzyme Complex IV. Collectively, these findings shift the paradigm on the contribution of individual cell types to the brain pathology of mitochondrial disorders.

Next, I used these mouse models to test therapeutic approaches for modulating mitochondrial dysfunction. The efficacy of two treatment approaches was evaluated in modulating the brain pathology of mice with astrocytic mitochondrial dysfunction. Both strategies acted to remodel cellular metabolism, but through different mechanisms. The first intervention used rapamycin to inhibit activity of the key nutrient sensor mTORC1; while the second used dietary intervention by shifting the carbon source to generate ketone bodies as an alternative energy source for the brain. Neither of the treatments improved the spongiotic pathology or attenuated reactive astrogliosis, and moreover the ketogenic diet exacerbated these phenotypes. Since rapamycin and ketogenic diet have been used successfully in treating other mouse models of mitochondrial dysfunction, it emphasizes the importance of using disease-specific models in preclinical studies.

In the final part of my thesis, astrocyte responses to mitochondrial dysfunction were investigated. We found that lipid biosynthesis was downregulated in astrocytes, which was paralleled by changes in brain lipid composition and accumulation of lipid droplets. In contrast, mitochondrial dysfunction in neurons did not remarkably affect brain lipid composition. Finally, we discovered an induction of a motile ciliogenesis program as an astrocyte response to pathological stimuli. Mitochondrial dysfunction resulted in anomalous expression of motile cilia components and abnormal morphology of cilia in astrocytes. Astrocytes are normally devoid of motile cilia but possess a primary cilium, which has signalling functions. Our findings raise the possibility of the remodelling of cilia function in astrocytes in response to mitochondrial dysfunction, which may contribute to pathogenesis.

Altogether, the research presented in this thesis has implicated astrocytes as a critical contributor to mitochondrial disease manifestations, and provided a solid base for the future efforts to target astrocyte responses to mitochondrial dysfunction.

TIIVISTELMÄ

Mitokondriot ovat soluorganelleja, joilla on oleellisen tärkeä rooli energia-aineenvaihdunnan säätelyssä, ja mitokondrioiden toimintahäiriöt aiheuttavatkin joukon erilaisia sairauksia. Tällaisia ovat muun muassa vakavat, kuolemaan johtavat lapsuusiän mitokondriaaliset aivosairaudet, joihin ei tällä hetkellä ole hoitoa. Tyypilliset patologiset löydökset mitokondriaalisissa aivosairauksissa käsittävät aivojen hermoverkon rappeuman, sekä lisäksi muiden solutyyppien rakenteellisia muutoksia, erityisesti reaktiivista glioosia. Hermosolujen erityistä herkkyyttä mitokondrioiden toimintahäiriöille on perinteisesti pidetty pääasiallisena syynä tautien ilmenemiseen, ja reaktiivista glioosia hermotuhon toissijaisena seurauksena.

Väitöskirjassani olen tutkinut eri solutyyppien osuutta keskushermostollisten mitokondriaalisten toimintahäiriöiden taustalla. Kuten odotettua, hermosolut olivat herkkiä mitokondrioiden toimintahäiriölle. Astrosyyttisissä mitokondriopatologiamallissa taas havaitsimme, että mitokondrioiden toiminnasta kärsivät solut eivät kuolleet, vaan ilmensivät itse aktiivista reaktiivista astroglioosia. Näiden hiirten aivoissa havaitsimme lisäksi spongioottisille enkefalopatioille tyypillistä, pesusienimäistä rakkularakennetta, jota esiintyy osalla mitokondriotautipotilaista. Yhdessä nämä löydökset muuttavat käsitystämme erillisten keskushermoston solutyyppien osallisuudesta mitokondriaalisten aivosairauksien patologiassa.

Seuraavaksi halusimme tutkia, voisiko joillakin mitokodnriosairauksiin jo aiemmin kokeilluilla terapiamuodoilla vaikuttaa hiirimalliemme patologisiin löydöksiin. Ensimmäinen interventio hyödynsi rapamysiiniä, jonka tiedetään hillitsevän solun ravinnetilannetta aistivaa mTORC1 proteiinia. Toinen hoito keskittyi ravintokoostumuksen kautta tuottamaan aivoille runsaasti ketoaineita energialähteeksi. Kumpikaan hoidoista ei kuitenkaan pystynyt korjaamaan rakkulamuodostusta eli spongioosia, eikä hillinnyt reaktiivista astroglioosia. Molemmat hoidot ovat aiemmin osoittautuneet lupaaviksi menetelmiksi hoitaa oireita toisilla mitokondriotautimalleilla, joten meidän tutkimuksemme korostaa tautispesifisten malliorganismien käytön tärkeyttä kliinisiä tutkimuksia suunniteltaessa.

Väitöstutkimukseni viimeisessä osassa halusimme perehtyä astrosyyttien reaktioihin mitokondrioiden toimintahäiriötilanteessa. Huomasimme, että mitokondriopatologien seurauksena lipidien synteesi oli huomattavasti vähentynyt astrosyyteissä, vaikka samaan aikaan hiiren aivoihin kertyi lipidipisaroita. Lisäksi havaitsimme, että mitokondriopatologia johti solunulkoisten liikkuvien karvojen, eli motiilien siilioiden muodostumiseen astrosyyteissä. Löytämämme epätyypillinen siilioiden muodostusohjelma käsitti sekä voimakkaan geeni-ilmennyksen että muuttuneen morfologian. Normaalisti astrosyytit eivät ilmennä liikkumisessa käytettäviä siilioita, mutta niillä on viestinnässä käytettävä niin sanottu primaari-siilia. Löydöksemme osoittaa, että rasva-aineenvaihdunta ja siilioiden muodostus astrosyyteissä saattaa olla osa patogeneesiä mitokondrioiden toimintahäiriöissä.

Yhteenvetona, tässä työssä esitetty tutkimus on nostanut astrosyyttien toiminnan ja niiden häiriöt entistä tärkeämpään osaan mitokondriotautien patologiassa. Väitöskirjassani esitän uusia löydöksiä, jotka luovat pohjan tuleville astrosyyttisiin vasteisiin keskittyville tutkimushankkeille mitokondriotaudeissa.

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Helsinki, May 2021
Olesia Ignatenko

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their roman numerals.

I) O. Ignatenko*, D. Chilov*, I. Paetau, E. de Miguel, C.B. Jackson, G. Capin, A. Paetau, M. Terzioglu, L. Euro, A. Suomalainen. Loss of mtDNA activates astrocytes and leads to spongiotic encephalopathy. Nature Communications 2018. DOI : 10.1038/s41467-017-01859-9). * = equal contribution.

II) O. Ignatenko, J. Nikkanen, A. Kononov, N. Zamboni, G. Ince-Dunn, A. Suomalainen. Mitochondrial spongiotic brain disease: astrocytic stress and harmful rapamycin and ketosis effect (Life Science Alliance 3 (9). DOI: 10.26508/lsa.202000797).

III) O. Ignatenko, S. Malinen, J. Nikkanen, A. Kononov, H. Vihinen, E. Jokitalo, G. Ince-Dunn*, A. Suomalainen*. Mitochondrial dysfunction remodels the ciliogenic program in astrocytes (Manuscript). * = co-corresponding author.

In addition, unpublished results are presented.

ABBREVIATIONS

ACSA-2	astrocyte cell surface antigen-2
ADP/ATP	adenosine diphosphate/adenosine triphosphate
CNS	central nervous system
CoA	coenzyme A
Cox10KO(astro)	*Cox10* knockout (in astrocytes)
CreER	Cre recombinase fused to the estrogen receptor
Ctrl	control
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
e.g.	from Latin exempli gratia, 'for example'
FAD(H_2)	flavin adenine dinucleotide (reduced)
FC	fold change
HEP	humane endpoint
i.e.	from Latin id est, 'that is'
i.p.	intraperitoneal injection
ISR	integrated stress response
ISR^{mt}	mitochondrial integrated stress response
MIP	maximum intensity projection
mo	months
mtDNA	mitochondrial DNA
mTorc1	mammalian target of rapamycin complex 1
NAD(H)	nicotinamide adenine dinucleotide (reduced)
OXPHOS	oxidative phosphorylation
PBS	phosphate-buffered saline
PCA	principal component analysis
PFA	paraformaldehyde
(RT-q)PCR	(reverse transcription-quantitative) polymerase chain reaction
RNA, mRNA, rRNA, tRNA	ribonucleic acid (messenger, ribosomal, transfer)
RT	room temperature
TF	transcription factor
TwKO(astro/neuro)	*Twnk* knockout (in astrocytes/neurons)

1 INTRODUCTION

The brain has a complex cellular organisation, and is composed of highly specialised cell types that diverge in function, morphology, and molecular composition. Major cell populations of the brain include neurons, astrocytes, oligodendrocytes, oligodendrocyte progenitor cells, and microglia. Integral to the tissue are also cells that compose vasculature and brain-fluid barriers. In the mammalian brain, the co-existence of these cell populations forms a highly intermingled, dynamic three-dimensional network.

Inherited brain diseases are caused by pathogenic DNA variants that can impede homeostasis and intercellular communication of every brain cell population. The pathogenic manifestations at the cellular level can however differ dramatically. Well known brain diseases include Alzheimer's, Parkinson's, and Huntington's disease, all of which are defined as neurodegenerative, as they manifest with degeneration of neuronal cells. Non-neuronal cells, notably astrocytes and microglia, present with changes in morphology, gene expression and function, collectively referred to as reactive gliosis. Such changes were historically attributed as secondary to the neuronal pathology.

Mitochondria are fundamental eukaryotic organelles, the functions of which are indispensable for cell homeostasis. Pathogenic DNA variants that affect protein function in mitochondria cause an array of human diseases, defined as mitochondrial diseases. It would be plausible to assume that the homeostasis of each cell type in the central nervous system may be perturbed, contributing to pathogenesis. Research efforts have thus far concentrated on the pathology arising from mitochondrial dysfunction in neurons. This established the concept of neuronal vulnerability to mitochondrial dysfunction, but may have overlooked the contribution of other cell types.

In this thesis, I present the experimental data to which I contributed personally. The research presented in this thesis is based upon several manuscripts to which multiple authors contributed, hence the personal pronoun 'we' is used throughout. In the research presented in this thesis, we investigated the contribution of astrocytes to the pathogenesis of mitochondrial dysfunction in the central nervous system (CNS). Using genetically modified mouse models, we induced conditional genetic knockout of genes *Twnk* and *Cox10,* which encode essential mitochondrial proteins. We found that mitochondrial dysfunction in astrocytes is sufficient to drive brain pathology and cell responses that are observed in human diseases. This implicated astrocytes as a critical contributor to the pathogenesis of mitochondrial dysfunction in the CNS. Since mitochondrial dysfunction is a hallmark of common neurodegenerative diseases, this research is also relevant to consider when investigating pathologies manifesting with secondary mitochondrial dysfunction.

2 REVIEW OF THE LITERATURE

2.1 Cellular organisation of the mammalian brain

Paramount to nearly all multicellular animal life is the nervous system, which collects information from the surroundings and from the body itself, interprets and transmits it, and exerts commands over the entire organism. As early as the second century BC, Galen proposed that there is no distinction between the mental and the physical, and that human life is controlled by the nerves originating from the brain and the spinal cord. Fascination with the complexity of human cognition, and with life itself perhaps, will forever inspire alternative explanations. The ever accumulating reports of how brain diseases result in the disruption of all known cognitive processes, behaviors and motor functions (for example, refer to (Sacks 1995)), have provided us with an overwhelming body of evidence on the central role of the nervous system as the coordinating center of our life, laying the foundation of modern neurology and neuroscience.

Cells were first observed in the middle of the 17th century, and within a hundred years were recognised as the fundamental blocks of life. It was not until the mid 20th century that neuroscientists agreed that this principle also applied to nervous tissue (reviewed in (Shepherd 2015)). Early investigations into the architecture of the mammalian brain were challenging, because the visualisation techniques available at the time could not resolve the extreme organ complexity in order to establish cells as the principal units of nervous tissue (reviewed in (Bentivoglio et al. 2019; Glickstein 2006; De Carlos and Borrell 2007)). In the 19th century, Camillo Golgi developed a new staining approach that allowed visualisation of nervous system components with unprecedented clarity.

Consequently, Golgi promoted the reticular theory, postulating that the brain consisted of a continuous nerve network. However, using essentially the same technique, Santiago Ramón y Cajal concluded that the fundamental blocks of the brain were discrete cells. This view was later coined as the neuron doctrine. The spirit of this debate is reflected in the scientists' own words after sharing the Nobel Prize in 1906 for their work on the structure of the nervous system. In his Nobel lecture, Golgi still postulated that the neuron doctrine championed by Cajal was a fad already going out of favor (Finger 2004), while Cajal later commented: *"What a cruel irony of fate to pair, like Siamese twins united by the shoulders, scientific adversaries of such contrasting character!"* (Chu 2006). Shortly after, investigations into simpler nervous systems, like that of jellyfish, provided another line of evidence supporting the role of individual cells. The cell theory was finally confirmed for organisms of all complexities in the 1950s by electron microscopy, as it became apparent that projections of individual neuronal cells are not fused together. Over 500 million years, evolution has given rise to an impressively diverse array of animal nervous systems, and yet commonalities in cellular composition allow us to infer unifying functional principles (reviewed in (Martinez and Sprecher 2020)). From here on, I will however primarily focus on the mammalian brain, with an emphasis on the human and mouse brain that are most relevant for the research presented in this thesis.

2.1.1 The brain plan

The brain is a complex organ, often referenced as the most complex biological organ or system. Approaches to investigating complex systems commonly include studying a system through its simpler components, or reductionism. Applicable to the brain, reductionism has been proven to be extremely useful. Most commonly, brain biology is addressed at the level of distinct functions, regions, circuits, and cell types, the latter being the most relevant for the research presented in this thesis. Cell types are populations of cells that share a set of parameters such as morphology, function, and ontogeny. Initially, cell types in the brain were defined by distinct morphological features. This was followed up by functional investigations that confirmed a heuristic assumption that cells that look the same generally perform the same set of functions. Advancements in developmental biology also shed light on cell ontogeny and molecular machinery that control cell fate determination. Instrumental for cell-specific investigations was also the identification of the proteins that are enriched in a given cell type compared to others, referred to as cell-specific markers. These proteins may play important roles in cell-defining functions, cell differentiation and maintenance of cell type identity, or alternatively to display cell-specific expression in the absence of such functions. The advancement in single-cell analyses, most notably approaches using RNA sequencing, now allows characterisation of cell-specific profiles in unprecedented detail (reviewed in (Armand et al. 2021)), potentially leading to defining an almost infinite number of cell states (reviewed in (Trapnell 2015; Morris 2019)). However, the introduction of the major, classically defined, resident cell populations of the brain is most relevant to this thesis.

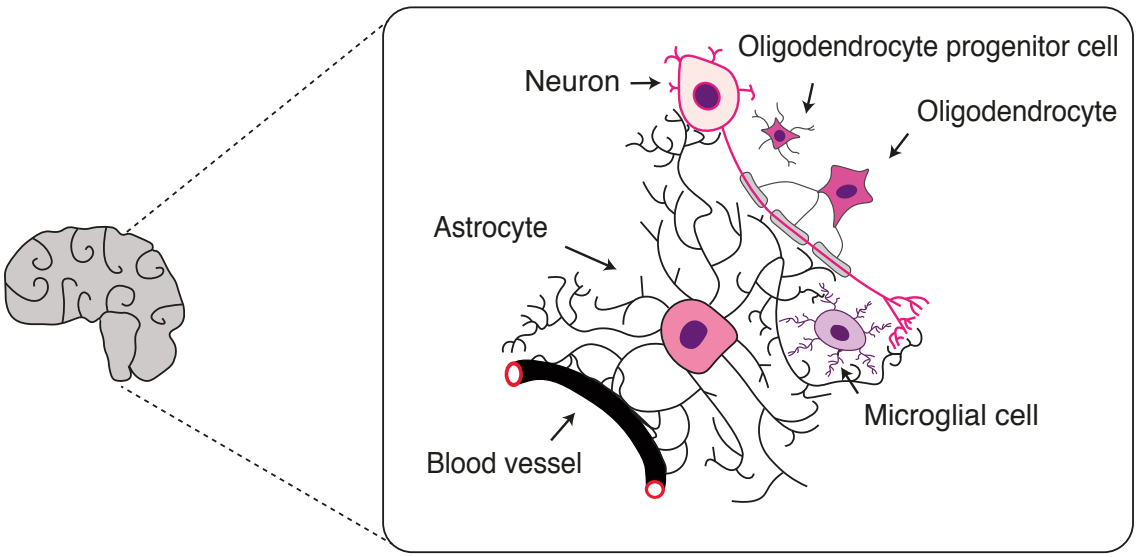

Figure 1: Schematic of the main resident cell types of the mammalian brain.

Major cell types residing in the adult brain are schematically depicted in Figure 1. Neurons are primarily involved in information processing and transmission, therefore performing the definitive functions of the nervous system. Astrocytes are integral to the function of the nervous tissue, and possess manifold functions including regulation of water and ion balance, synapse modulation, metabolite distribution, and blood-brain barrier property. Oligodendrocytes form intricate contacts with neurons, and produce myelin membrane wrapping around neuronal projections to facilitate impulse transmission.

Oligodendrocyte progenitor cells have potential to differentiate into oligodendrocytes throughout adulthood. Microglia are the resident macrophage population of the brain that mediate inflammatory responses, clear debris, and contribute to synapse formation dynamics. Ablation of neurons, astrocytes, or oligodendrocytes in rodents results in severe pathologies and shortened lifespan (reviewed in (Jäkel and Dimou 2017)). Ablation of microglia is asymptomatic in a healthy adult brain (although it can modify disease phenotypes), but is fatal during development (reviewed in (Jäkel and Dimou 2017)). These experiments signify that all of the major cell types play vital functions in brain homeostasis.

To understand how the complexity of brain cell composition is formed, here I give a simplified introduction to brain development through cell type specification (based on the following review articles: (Götz and Huttner 2005; Martínez-Cerdeño and Noctor 2018; Rowitch and Kriegstein 2010; Rowitch 2004; Ginhoux and Prinz 2015; Bilimoria and Stevens 2015; Miller and Gauthier 2007)). Upon fertilization and zygote formation, early events of embryonic development lead to formation of the three principal cell layers that give rise to the organ systems: ectoderm (the outer layer), mesoderm (the middle layer), and endoderm (the inner layer). A portion of the outer cell layer differentiates into cells of the neural lineage, or neuroepithelium. This rather homogeneous population of cells will give rise to the myriad of neuronal types, astrocytes, oligodendrocytes, and their progenitors. Microglial cells however have a distinct origin. The principles of cell type differentiation presented below are most extensively studied for the cerebral cortex.

Neuroepithelial cells expand to generate a plate of cells, in the middle of which a groove is formed. This groove deepens, leading to convergence of proximal parts of the neuroepithelial plate and resulting in the formation of a hollow tube filled with embryonic cerebrospinal fluid, known as neural tube. Further differentiation of the neural tube advances to form bulge-like parts, each eventually giving rise to specific regions of the nervous system. From the dorsal part, the cerebellar cortex is formed. The pool of neuroepithelial cells facing the cavity of the neural tube continues to expand, forming a ventricular zone. Neuroepithelial cells residing there acquire characteristic changes in morphology and gene expression, by which these cells become defined as radial glial cells. Radial glial cells typically possess two long processes, with endfeet protruding to the ventricular surface and to the pia mater. Radial glial cells divide symmetrically to produce self-renewing radial glial cells; or asymmetrically, to produce a neuronal precursor cell and a radial glial cell. Radial glial cells share a number of characteristics with mature astrocytes, and are referred to as a glial cell subtype, or even an astrocyte subtype.

Neuronal precursors transform into neurons and migrate to populate the developing brain. This process is tightly regulated spatiotemporally, as neurons that populate specific brain areas are generated in an orderly manner. Many of these committed neural precursors use the long projections of the radial glial cells as a scaffold during migration. The first waves of neurogenesis are followed by the generation of the astrocyte precursors from a portion of radial glial cells. Most astrocyte precursors migrate to the cortical plate during embryogenesis (Clavreul et al. 2019; Ge et al. 2012). These cells expand locally during early postnatal development, exit the cell cycle, and transform into mature astrocytes. Oligodendrocyte progenitor cells are also generated during embryonic development from radial glia. These precursors migrate to populate the entire developing brain and differentiate locally into oligodendrocytes. The most active period of myelination is during postnatal development. Major myelin tracks are formed in defined periods during early postnatal development,

however myelination is not considered completed until adolescence. Myelination also continues throughout adulthood, contributing to neuronal plasticity and learning experience (Hughes et al. 2018).

In contrast to the cell types discussed above, microglial cells do not originate from radial glial cells, and therefore have a distinct origin from neurons, astrocytes, and oligodendrocyte lineage cells. Microglia originate from yolk sac myeloid precursors and independently populate the developing brain during early embryogenesis. The distinct developmental origin of microglia has provoked debate to ostracise the cell type from a glial classification. However, considering how integral microglia are to the nervous tissue, this would appear to be little more than a semantic exercise.

After development is completed, the brain retains only a limited capacity to generate neurons. Neurons are postmitotic and cannot divide. Adult neural stem cells are radial glia-like (or astrocyte-like) cells that reside in the subventricular area and in the dentate gyrus of the hippocampus. These cells can generate hippocampal and olfactory bulb neurons, which is shown to be continuous at least in rodents. So far, there is no convincing evidence of the generation of other neuronal types, nor astrocyte cell division, or their differentiation from other progenitors in the mature healthy brain. Most of the neurons and astrocytes generated during postnatal development might therefore be finite for the brain. In turn, a proportion of oligodendrocyte progenitor cells continues to divide and generate oligodendrocytes throughout adulthood (Rivers et al. 2008; Hughes et al. 2013, 2018). Consistent with this, oligodendrocyte lineage in the adult brain is complex, and comprises cells at various maturation stages (S. Marques et al. 2016; Spitzer et al. 2019). Microglial cells are slow-cycling in the healthy adult brain, but can proliferate in disease and replenish the cell pool after its almost complete elimination (Elmore et al. 2014). Astrocytes also can re-enter the cell cycle in a disease setting, such as traumatic injury and stroke (further discussed in 2.2.1.2).

2.1.2 Non-astrocytic cells

(Neuro)science. Each major cell population of the brain populates the entire parenchyma. However, the nervous system is defined by the presence of neurons. This is not explained by neurons being discovered much earlier than non-neuronal cells, as other cell types were already depicted in the works of Cajal, and suggested by him to play important roles. In 1856, Rudolf Virchow termed non-neuronal cells of the brain as 'glia' (from the ancient Greek for 'glue'), reflecting their evident contribution into forming the brain structure. So, why 'neuro'?

Already in the 18th century, it was demonstrated that motor functions could be perturbed by the disruption of nerves, which were later discovered to be projections of the neural cells (Bear, Connors, and Paradiso 2001). This provided important evidence that neurons directly control major properties of the body. Since muscles were responsive to electrical stimulation, it was also established that an ability to perceive and transmit electrical impulses might be the key to motor processes. Later, it turned out that neurons, unlike any other known cells, were responsive to electrical stimuli and were able to transmit these signals. This was followed by the understanding that to process specific kinds of information in a directed manner, neurons form assemblies, or neural circuits. A simple example of a neural circuit is a knee reflex, where information from sensory neurons is passed via intermediate neurons onto motor neurons, which evoke movement. Further investigations kept demonstrating how other functions of the nervous system are also controlled by neural circuits. Collectively, it was established that propagation of the electrochemical signals by neurons is

the key mechanism which underlies functions of the nervous system. Glial cells, on the other hand, were not initially demonstrated to engage in these processes. Arguably, for decades this acted as an important determinant of the neurocentric focus of the field.

Another 'neurocentric stimulus' for the field comes from investigations of human pathologies. It has been appreciated that in many human brain diseases neurons, unlike astrocytes or microglia, degenerate (further discussed in 2.2). It was commonly interpreted that to combat brain diseases requires the prevention or reversal of neurodegeneration, and therefore research efforts should be concentrated on neuronal biology. The last twenty years has seen a Renaissance of glial research, as glial cell populations were discovered and re-discovered to be indispensable for every function of the nervous system, including neural transmission (reviewed in (Allen and Barres 2009; Araque and Navarrete 2010; Dallérac, Chever, and Rouach 2013)). Below, I introduce each of the major cell populations in the adult brain, with the exception of astrocytes that are further discussed in 2.1.3.

Neurons. The nervous system is defined by the presence of neurons. These are excitable cells that propagate electrochemical signals along their projections. The frequency and duration of the electrochemical signals ultimately determine the message delivered by the neurons.

The ability to evoke and transmit electrochemical signals is derived from the specialised morphology and membrane properties of a neuronal cell (reviewed in (Bear, Connors, and Paradiso 2001; Takano et al. 2015)). At resting state, the neuronal membrane, like any other cell membrane in our body, has a negative potential. That is, the net sum of the ion charge inside the cell is lower than outside of the cell. Protein pumps embedded in the cell membrane regulate ion transport. Upon stimulus, ion channels change conformation, facilitating exchange of the ions with the outer space and leading to a change in the membrane potential, or membrane depolarisation. The stimulus is considered inhibitory if it leads to a further decrease of the membrane potential, or excitatory, if it leads to an increase of the membrane potential. Stimuli are integrated at the cell soma by the branched membrane extensions called dendrites. A typical neuron also possesses a long projection (called an axon), capable of propagating the electrical signal and passing it onto the next neuronal cell(s) in the chain. This occurs if the influx of positively charged ions reaches a critical threshold, triggering a spike-like change of the membrane potential called an action potential. An action potential triggers the opening of a sequence of ion channels, progressing in a cascade along the axonal membrane. To pass an electrical signal onto a neighboring cell requires an intermembrane channel allowing for ion exchange (electrical synapse), or the presence of a molecular machinery which will transmit an electrical signal into a chemical signal, in turn triggering a response from a neighboring neuron (chemical synapse). In the adult mammalian nervous system, synapses are predominantly chemical. That is, chemical messengers are stored in synaptic vesicles in the axonal terminals. Once an action potential arrives at these terminals, it triggers an influx of Ca^{2+} ions that can then interact with a set of proteins that stimulate fusion of the synaptic vesicles with the cell membrane of the axonal terminal. Messenger compounds are released into the synaptic cleft, and interact with receptors on the membrane of a perceiving neuron, causing a shift in its membrane polarisation. As the function suggests, chemical messengers are known as neurotransmitters, and the two participating in the signal transmission neurons are called pre- and post- synaptic. As a general approximation, neurotransmitters and synapses can be classified as inhibitory or excitatory, depending on the direction of the membrane potential change evoked.

Our brain is estimated to comprise over 80 billion neurons (Lent et al. 2012; Azevedo et al. 2009). In development, an excess number of neurons and synapses are generated. Postnatal development includes eliminating excess neurons and synapses, the latter termed synaptic pruning. Throughout life, the nervous system retains the ability to undergo a dynamic remodelling process, known as neural plasticity. This includes both the dynamics at the synaptic level and the modulation of neural connections and networks. Together, neural plasticity underlies many of our cognitive functions, including learning.

Neurons are an extremely heterogeneous population of cells. Classifications are based on a set of properties, such as function, morphology, a dominant type of the neurotransmitter released, location of the axon within one region or its projection outside, and many more.

Oligodendrocytes enwrap extensions of their cell membrane around neuronal axons in concentric fashion, forming myelin sheaths (reviewed in (El Waly et al. 2014)). A single oligodendrocyte may extend myelinating projections to dozens of axons. Thus, the cell membrane of a pre-myelinating cell undergoes an enormous expansion (reviewed in (Chrast et al. 2011)). Myelin acts as insulation to facilitate directional propagation of action potentials in a saltatory (or jumping) manner between myelin segments, which are separated by unmyelinated gaps with exposed axolemma, called Nodes of Ranvier (schematically depicted in Figure 1). This mechanism allowed for the increased complexity of neural networks throughout evolution, while keeping the diameter of axons constrained. Additionally, it has been argued that oligodendrocytes also provide axonal support independent of myelination (reviewed in (Philips and Rothstein 2017; Simons and Nave 2015)). A portion of axons is myelinated across the entire parenchyma. Myelinated axons also form tracts that connect different brain regions to one another. Myelination is essential for neuronal functions, and myelin disruption is associated with various pathologies, including those leading to fatal and debilitating outcomes.

Oligodendrocyte progenitor cells are a population of cells that can differentiate into oligodendrocytes. These cells express neuron-glial antigen 2 (NG2), and are also known as *NG2$^+$ cells* (Larson, Zhang, and Bergles 2016). In a healthy adult brain, generation of new myelinating oligodendrocytes by NG2$^+$ cells contribute to experience-dependent myelination and neural plasticity (Hughes et al. 2013, 2018; Bacmeister et al. 2020). Demyelination that occurs in a disease setting may induce NG2$^+$ proliferation and differentiation, which contributes to remyelination (Tripathi et al. 2010; Di Bello et al. 1999; S. H. Kang et al. 2010). NG2$^+$ cells express neurotransmitter receptors, and change membrane potential upon receiving synaptic inputs from neurons. This was established to regulate the cell fate of NG2$^+$ cells (Gibson et al. 2014). Other functional outcomes of these neuron-NG2$^+$ intercellular communications are not yet clearly elucidated, however these atypical properties for glial cells evoke interest.

Microglia function as the immune cells of the brain that detect pathogen invasions and brain damage, have phagocytic and cytotoxic activities, and are also essential for synapse pruning (reviewed in (Hong and Stevens 2016)). Microglia display a variety of responses in their ever-changing environment, possessing motile projections even in the healthy brain. These cells are capable of migration and cell division, most typically stimulated by pathogens or lesions. Adult microglia may appear uniform morphologically, but the versatility of this cell population is reflected by the presence of distinct subpopulations and cellular states throughout adulthood (Hammond et al. 2019).

The other 'glue'. To nourish brain cells, to remove waste products, and to provide a reservoir for interstitial fluid formation, the brain requires systems that distribute blood and cerebrospinal fluid (reviewed in (Profaci et al. 2020; Shetty and Zanirati 2020)). Blood in the brain circulates through a vast vascular network. Cerebrospinal fluid bathes the inside of the brain through a system of cavities known as ventricles, and the outside of the brain in between the protective layers known as meninges. To provide a controlled ionic and biochemical environment for parenchymal brain cells, the exchange of molecules between the interstitial space and brain fluids is tightly regulated. These filtering properties are called barriers, of which here are discussed the blood-brain, cerebrospinal fluid-brain, and blood-cerebrospinal fluid barriers (reviewed in (Profaci et al. 2020; Liddelow 2011; Redzic 2011; Jiménez et al. 2014)). The role of astrocytes in the function of these barriers is discussed in 2.1.3.5.

The blood-brain barrier is a concert of properties that limit the diffusion of blood-borne molecules from the vasculature system to the brain milieu (reviewed in (Profaci et al. 2020)). Central to the blood-brain barrier is the membranes of brain vascular endothelial cells, which are connected by tight junctions. These junctions prevent the diffusion of water-soluble molecules between the cell membranes, enforcing a transcellular route of transport. Lipophilic molecules are able to pass through without requiring an active transport, which is however limited by the action of efflux protein machinery (reviewed in (Dallas, Miller, and Bendayan 2006; Löscher and Potschka 2005)).

The blood-cerebrospinal fluid barrier is formed in the areas where the vasculature of several ventricular structures forms an exception to a classical blood-brain barrier organisation. The epithelium of such vessels partially lacks tight junctions and is therefore fenestrated, resulting in the leakage of tracing molecules and blood from these vessels. The barrier function is transferred to a specialised population of glial cells that form the outer layers of ventricular structures and possess tight junctions. These cells are known as ependymal cells. As the basal membrane of these cells contacts ventricular cerebrospinal fluid, the resulting barrier is a blood-cerebrospinal fluid barrier. The major blood-cerebrospinal fluid interface is positioned at the surface of a branched, highly vascularised and folded ventricular tissue called the choroid plexus (reviewed in (Liddelow 2011, 2015)). Other blood-cerebrospinal fluid interfaces are positioned at ventricular formations known as circumventricular organs (reviewed in (Miyata 2015)). These include those surrounding the hypothalamus, providing a mechanism of blood-to-brain hormonal signalling.

Finally, a cerebrospinal fluid-brain barrier is formed at the main sites of cerebrospinal fluid allocation: brain ventricles. Brain ventricles are carpeted by ependymal cells that possess modified tight junctions allowing limited diffusion, and such forming a partial barrier (reviewed in (Jiménez et al. 2014)). Ependymal cells are further discussed in 2.1.3.5.

2.1.3 Astrocytes

Astrocytes ('star-shaped' cells) are key for maintaining CNS homeostasis. They populate the entirety of brain parenchyma and engage in intimate interactions with one another and with every other major cell population in the brain (Figure 1). Astrocytes maintain a viable environment in the brain, as these cells control the osmotic and neurotransmitter balance, contribute to blood-brain and blood-spinocerebellar fluid barriers, shape synapse formation and function, interact with immune cells, and provide metabolites to other cells types (reviewed in (Sofroniew and Vinters 2010; D. D. Wang and Bordey 2008)). Astrocytes display a degree of diversity across the brain and within a given region at the morphological (Figure 2),

functional (reviewed in (Khakh and Deneen 2019)), as well as transcriptomic levels (Lozzi et al. 2020; Bayraktar et al. 2020; Batiuk et al. 2020). Below, I introduce the basic characteristics of astrocytes and some specific functions. The role of astrocytes in disease is discussed in 2.2.1.

2.1.3.1 Holistic importance of astrocyte function

The importance of astrocytes in the nervous system is supported by evolutionary evidence. Glial cells were not found in Cnidarians, but astrocyte-like cells exist in simple invertebrates and all organisms with nervous systems of higher complexity (reviewed in (Freeman and Rowitch 2013; Verkhratsky, Ho, and Parpura 2019; Verkhratsky and Nedergaard 2016)). In invertebrate model organisms *Caenorhabditis elegans* and Drosophila, astrocyte-like cells interact with sensory neurons, sculpt synapses and neurite outgrowth, modulate the environment, and form interactions analogous to blood-brain barrier connections (reviewed in (Freeman and Rowitch 2013)). These functions are reminiscent of astrocytes in the mammalian brain. Generally, increased brain complexity is accompanied by an increased proportion, diversity, morphological complexity, and cell volume of astrocytes in the nervous tissue (reviewed in (Allen 2014; Verkhratsky, Ho, and Parpura 2019; Maiken Nedergaard, Ransom, and Goldman 2003)). The functional evidence comes from the fact that ablation of astrocytes is incompatible with normal nervous tissue function; in mice this leads to neurodegeneration, motor impairment, and paralysis (Cui et al. 2001; Schreiner et al. 2015). Collectively, this shows a tight cooperation between astrocytes and neurons, and frames astrocytes as an integral component of the nervous tissue.

2.1.3.2 Morphology

Since the 19th century, the main morphological types of astrocytes have been defined as i) protoplasmic, spherical ramified cells with numerous radial projections residing mostly in grey matter; and ii) fibrous, less branched cells with long processes, residing mostly in the white matter (Andriezen 1893) (Figure 2A). Cerebellar cortex possesses two more morphologically distinct astrocyte populations: i) Bergmann glia, which make rosettes around some of the most branched neurons in our brain, Purkinje cells and ii) velate cells, protoplasmic-like astrocytes that ensheath individual neurons in the molecular layer (reviewed in (Hoogland and Kuhn 2010)).

Fibrous astrocytes possess long, relatively unbranched, processes (Figure 2A). The terminal ends of these processes often contact axons at nodes of Ranvier, however the functional coupling at these contacts is not well elucidated (Ffrench-Constant et al. 1986; Butt, Duncan, and Berry 1994). Protoplasmic astrocytes are globular cells that possess several primary and secondary branches which divide to numerous finer processes and branchlets, forming a bushy, sponge-like structure (Figure 2A). Such morphology is reflected in the original anatomical description by Mihály Lenhossék, who named astrocytes 'spider cells', or spongiocytes (Lenhossék 1893). Astrocytes occupy largely non-overlapping domains in brain parenchyma (the organisation principle commonly referred to as tiling) (Bushong et al. 2002; Halassa et al. 2007). The development of vast astrocyte branching appears to require neuron-derived factors, rather than being a cell-autonomous property (Stogsdill et al. 2017; Stork et al. 2014).

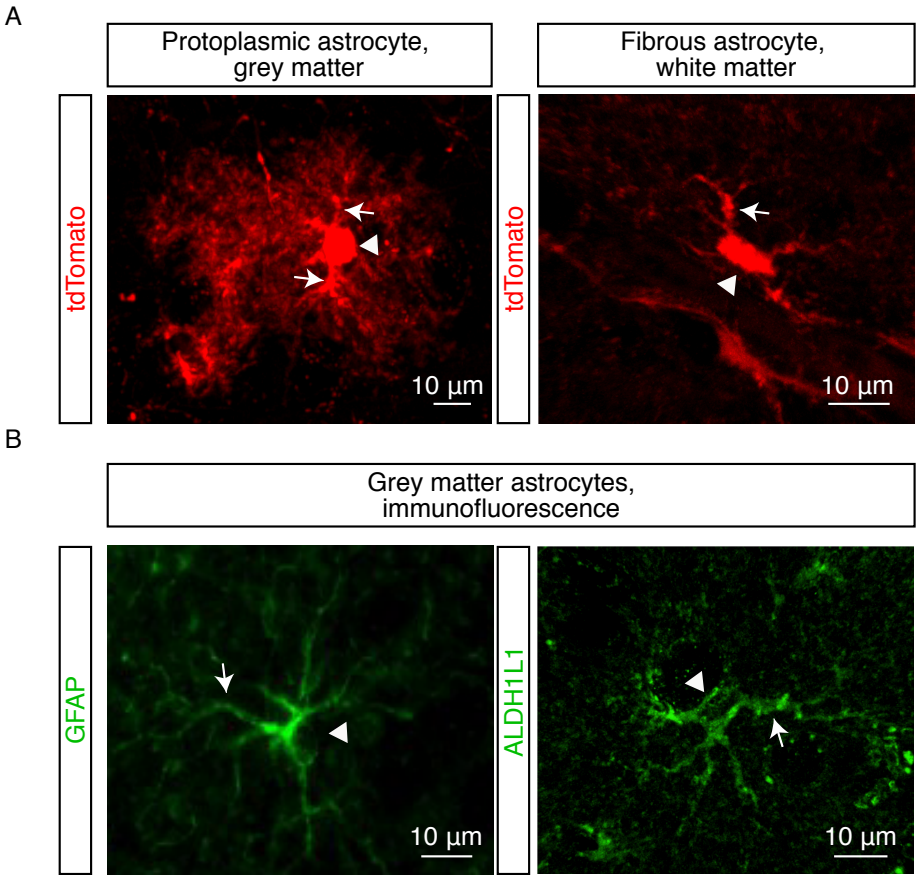

Figure 2. Astrocyte morphology in the mouse brain. Arrowheads mark cell soma, arrows mark primary and secondary branches of a cell.
(A): Cre recombinase-dependent expression of tdTomato protein with cytoplasmic localisation, Cre expression is driven by GFAP73.12 promoter (see 4.2). MIP, confocal images.
(B): immunofluorescence against GFAP (epifluorescence) and ALDH1L1 (MIP, a confocal image). ALDH1L1 imaging by Satu Malinen.

Importantly, immunostainings commonly used to visualise astrocytes, including a cytoskeletal glial fibrillary acidic protein (GFAP), typically only stain part of the cell and main cellular branches (Figure 2B). More detailed visualisation of astrocyte morphology is achieved with dye-filling techniques (Bushong et al. 2002) or by expression of membrane-targeted fluorescent proteins (Benediktsson et al. 2005). Cytoplasmic expression of fluorescent proteins also reveals a bush-like morphology in a fraction of the astrocytes (Figure 2A). An additional limit to examining the full extent of astrocyte morphology is that the finest cell processes are smaller than the resolution limit of light microscopy (reviewed in (Khakh and Deneen 2019)).

All aforementioned astrocyte classes appear in both the human and rodent brain, although human astrocytes are larger and more branched (reviewed in (Vasile, Dossi, and Rouach 2017)). The astrocytic transcriptome displays a high level of conservation between mice and humans (J. Li et al. 2020), and studies that dissected individual cell characteristics concluded that human and mouse astrocytes share the main functional and morphological features. A convincing evidence of this is also the fact that engrafts of human glial progenitors to a neonatal mouse brain or an adult spinal cord mature into astrocytes that functionally

integrate into mouse nervous tissue, resulting in significant replacement of mouse astrocytes (Hong Chen et al. 2015; Han et al. 2013). The human brain also possesses astrocyte populations unique to primate species with distinct morphology and positioning within cortical layers, called interlaminar astrocytes (Oberheim et al. 2009; Falcone et al. 2019; Colombo et al. 2005). The cell body of these cells resides in the first superficial layer of the cortex, either contacting the pia mater or not; with relatively unbranched tufts of cell processes descending through the underlying cortical layers. Since rodents do not have interlaminar astrocytes, the activities of these cells which may be potentially relevant for human disease cannot be seen when using mice as a model organism.

2.1.3.3 Astrocyte-astrocyte network

The outermost fine processes of neighboring protoplasmic astrocytes are highly intermingled (Wilhelmsson et al. 2006). Injection of a dye into one astrocyte results in its spread to the neighboring cells (Anders et al. 2014; Konietzko and Müller 1994). This observation points to physical coupling between astrocytes and is important conceptually, as it suggests that astrocytes might also exchange other molecules or ions.

The exchange of molecules between astrocytes is provided by transmembrane channel proteins called connexins (reviewed in (Giaume and McCarthy 1996; Houades et al. 2008)). Connexin subunits in the membrane of each adjacent cell form hemichannels, which together organise into ring-like channels known as gap junctions. Such intercellular coupling is not unique to astrocytes but widely presented across the cell types and tissues, and was originally discovered in the heart and liver (Revel and Karnovsky 1967). Gap junctions are permeable to ions, secondary messengers, and small molecules with molecular weight below 1000 daltons, providing a powerful mechanism of intercellular communication and metabolic support (Goodenough, Goliger, and Paul 1996). The selectivity and the extent of such exchange depend on the channel protein composition and the functional state of the cell (Weber et al. 2004). Partial disruption or ablation of astrocytic gap junctions led to abnormalities of varying severity, such as altered neuronal connectivity, vacuolation of brain parenchyma, demyelination, cell death, and sensorimotor deficits (Lutz et al. 2009; Theis et al. 2003; Frisch et al. 2003). Additionally, this disrupted the transport of lactate and glucose, providing evidence of the importance of gap junction coupling for the energetic balance (Clasadonte et al. 2017; Rouach et al. 2008). Astrocytes express numerous ion transporters and control the osmotic balance in the brain (reviewed in (Verkhratsky, Parpura, et al. 2019)). It is plausible that astrocyte coupling via gap junctions contributes to ion redistribution within the cellular network. Astrocytes also form heteromeric gap junctions with oligodendrocytes, suggestive of the coupling between these cell types (Orthmann-Murphy et al. 2007; Rash et al. 2001). Finally, it has been suggested that connexin hemichannels not paired to another cell might allow astrocytes to release metabolites to the extracellular space (Ye et al. 2003; Contreras and Sánchez 2002).

Astrocytes display transient elevations of intracellular calcium concentration, that can propagate along astrocytic processes and to neighboring cells (Szabó et al. 2017; Hirase et al. 2004; Newman and Zahs 1997; Dani, Chernjavsky, and Smith 1992; Agarwal et al. 2017; M. Nedergaard 1994). This may stimulate the release of molecules that display neurotransmitter activities (reviewed in (Haydon 2001; Bazargani and Attwell 2016; Volterra and Meldolesi 2005)), although this has been extensively debated in the field (reviewed in (Savtchouk and Volterra 2018; Sloan and Barres 2014; Maiken Nedergaard and Verkhratsky 2012; Fiacco and

McCarthy 2018)). Generally speaking, elevations in calcium concentration and its release outside of the cell are central to cell function and intercellular communication. Therefore, calcium signalling provides another piece of evidence of the functional coupling of astrocytes.

Astrocytic organisation is sometimes referred to as a syncytium (Scemes and Spray 2003; Kiyoshi and Zhou 2019). Formally, syncytium is a multinucleated cellular mass united by a continuous membrane, and astrocytes most definitely do not fall under this term. The physiological coupling can however be described as a 'functional syncytium', highlighting the complexity of organisation and function of this cell type.

2.1.3.4 Astrocyte-synapse contacts

Astrocyte processes extend to neuronal cell bodies and synapses (Ventura and Harris 1999). One simple piece of evidence of the importance of such interaction was that astrocytes, or astrocyte-conditioned media, increased the activity and the number of synapses in cultured neurons (Ullian et al. 2001; Pfrieger and Barres 1997). This could be mediated by either direct effects at the synapse or indirectly, such as by modulation of neuronal cell maturation and fitness. Further evidence of astrocyte function at the synapse came from discovering astrocytic uptake of neurotransmitters from a synaptic cleft, notably the glutamate uptake (Bergles and Jahr 1998; Rothstein et al. 1996; Voutsinos-Porche et al. 2003). Since then, numerous studies have added to the evidence that astrocytes secrete molecules modulating synapse formation and function (reviewed in (L. E. Clarke and Barres 2013; Allen and Eroglu 2017)), and display phagocytic activities at the synapse (J.-H. Lee et al. 2020; Chung et al. 2013). The extent of this interaction was highlighted by the discovery of over 100 proteins at the astrocyte-synapse interface *in vivo* (Takano et al. 2020). Neuronal activity also exerts transcriptional changes in co-cultured astrocytes and vice versa, indicative of the functional cooperation between these cells (Hasel et al. 2017).

It is commonly stated that a single mouse astrocyte can contact 100,000 synapses, and a human astrocyte can contact 2, 000, 000 synapses (L. E. Clarke and Barres 2013; Walker, Risher, and Risher 2020; Halassa et al. 2007; Allen and Eroglu 2017; Vezzoli et al. 2020; Oberheim, Goldman, and Nedergaard 2012; Agarwal and Bergles 2014). This number originates from an approximation of the astrocyte volume to an average density of the synapses in rat hippocampus (Bushong et al. 2002; Kirov, Sorra, and Harris 1999). Quantitative validation of this perhaps is beyond realistic, however it can be confidently stated that a single astrocyte indeed contacts numerous synapses (Kikuchi et al. 2020; Ventura and Harris 1999; Kiyoshi et al., 2021), and that this interaction is functionally important.

2.1.3.5 Astrocyte-barrier interactions

Blood-brain and blood-cerebrospinal fluid barriers prevent diffusion of molecules from circulating fluids to the brain interstitium. The function of these barriers is vital for the brain homeostasis, as it provides a controlled extracellular environment, regulating ionic and biochemical compositions (discussed in 2.1.2).

Astrocytic endfeet. Astrocytes possess polarised endfeet which enwrap blood vessels (a feature depicted in the 19th century (Andriezen 1893)), providing nearly complete coverage (Mathiisen et al. 2010) (Figure 3A). This is indicative of the role of astrocytes in the uptake and distribution of blood-derived metabolites including glucose, as such anatomy suggests that exchange of molecules with blood would require passing the astrocytic interface. The direct investigation of this is however challenging due to technical limitations in metabolite tracing.

Considering cerebrospinal fluid, tracer injection studies suggest that in brain parenchyma it is transported along the perivascular space formed by astrocytic endfeet, contributing to waste clearance (Iliff et al. 2012; Mestre et al. 2018).

Astrocytic endfeet are enriched in the water channel Aquaporin 4 (Figure 3A) and a potassium channel KIR4.1, both of which contribute to the control of osmotic balance. Gene ablation of either channels in mice however does not result in an overall brain pathology or affect organismal fitness, suggesting that this function is not essential at least in the short-term (Skucas et al. 2011; Papadopoulos et al. 2004; Manley et al. 2000; Neusch et al. 2006; Kucheryavykh et al. 2007; Mestre et al. 2018). The intimate interaction of astrocytes with blood vessels may also suggest the role of astrocytes for the blood-brain barrier property. Astrocytes indeed secrete multiple factors that induce blood-brain barrier formation and regulate blood flow (reviewed in (MacVicar and Newman 2015; Abbott et al. 2006; Michinaga and Koyama 2019)), and astrocyte depletion results in barrier leakage (Heithoff et al. 2021).

Ependymal cells. Ependymal cells form the surface of brain ventricles, possess gap junctions, and constitute cerebrospinal fluid-brain barrier (Figure 3, see also 2.1.2). Ependymal cells and astrocytes have progenitors and share a number of cell markers (Spassky et al. 2005; Jacquet et al. 2009). Ependymal cells posess tufts of motile cilia organelles, which protrude to the ventricular space (Figure 3B). The coordinated beating of ependymal cilia facilitates cerebrospinal fluid movement (García-Verdugo et al. 1998; Olstad et al. 2019). Large-scale movement of cerebrospinal fluid in the brain is regulated by the heartbeat, however the disruption of ependymal cilia motility invariably results in hydrocephalus (Raimondi, Clark, and McLone 1976; Ibañez-Tallon et al. 2004; Lechtreck et al. 2008; Olstad et al. 2019). Ependymal cells of the choroid plexus are also multiciliated and possess tight junctions, forming the blood-cerebrospinal fluid barrier (see 2.1.2). Another class of ependymal glia is tanycytes, which constitute surfaces of circumventricular organs (Langlet et al. 2013). Tanycytes form gap junctions but do not possess tufts of motile cilia. Tanycytes also extend a long process that comes in close contact with fenestrated blood capillaries, forming a blood-cerebrospinal fluid interface.

In summary, astrocytic endfeet form a specialised anatomical compartment in the brain, which appears to be important for metabolite distribution and waste clearance in brain parenchyma. Ontogenically related to astrocytes, ependymal cells constitute brain-fluid interfaces and present with unique anatomical and functional properties.

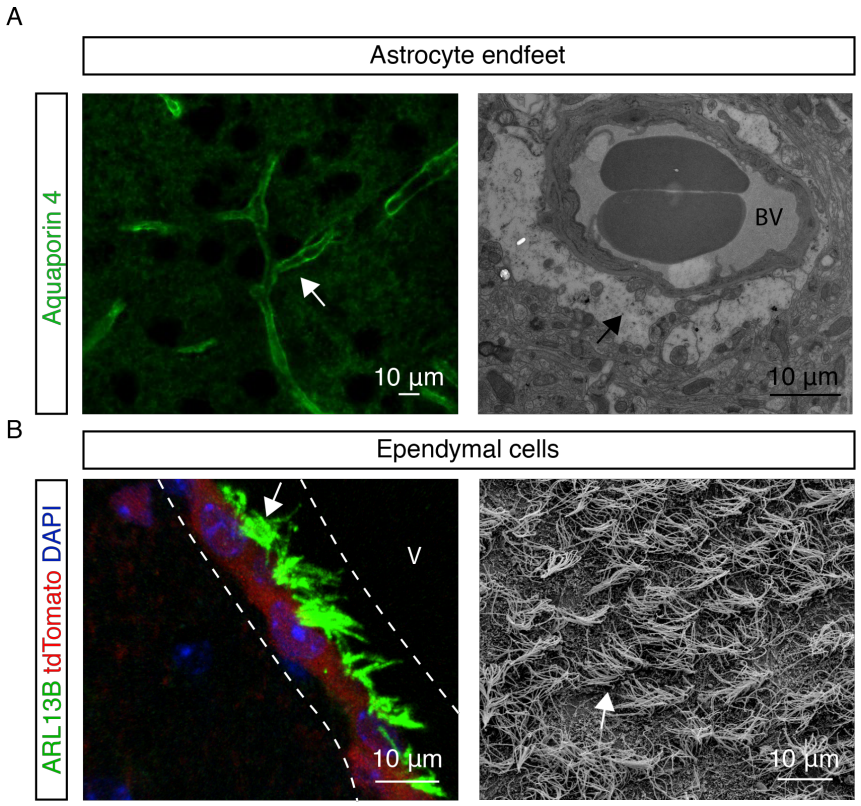

Figure 3. Astrocyte endfeet and ependymal cells, mouse brain. **(A):** Immunofluorescence against Aquaporin 4 (an epifluorescence image) and transmission electron microscopy. Arrows indicate the endfeet surrounding a blood vessel. BV = blood vessel. **(B):** Immunofluorescence against a component of ciliary axoneme ARL13B and Cre-dependent expression of tdTomato (MIP, a confocal image) and scanning electron microscopy. Arrows indicate tufts of ependymal cilia. Dashed lines indicate ependymal cell layer. V = ventricle. Counterstain with nuclear DNA (DAPI).

2.1.4 Metabolic cooperation between brain cells

As introduced above, cells in the brain interact anatomically and functionally. Cooperation of brain cell types may also include the exchange of metabolites.

2.1.4.1 The complexity of cellular metabolism

All processes of life are enabled by continuous synthesis and breakdown of small molecules. These occur through biochemical reactions, the sum of which is called metabolism. Linked series of chemical reactions comprise metabolic pathways, where a product of one reaction is a substrate of the next one.

Simply put, every cell requires the imported molecules to be used as an energy source and to generate its internal structures; while simultaneously exporting the products of its metabolism. Central pathways that convert nutrients into chemical energy and precursors of essential metabolites are: glycolysis (breakdown of glucose), **β**-oxidation (breakdown of fatty acids), and protein catabolism (breakdown of proteins) (Figure 4) (reviewed in (Nelson, Lehninger, and Cox 2008)). End products of these pathways, including acetyl-CoA, act as substrates for the tricarboxylic acid cycle, a series of reactions to release chemical energy and produce reducing agents (Figure 4). Specifically, the tricarboxylic cycle and **β**-oxidation

generate the reduced cofactors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) (Figure 4). These molecules are utilised in oxidative phosphorylation (see 2.3.2), resulting in the production of ATP, as well as oxidized cofactors NAD^+ and FAD^+ (Figure 4). To produce ATP and to maintain the ratio between oxidised and reduced forms of those essential cofactors requires coordinated function of the central metabolic pathways (Figure 4). Cells also store energy as polymers of glucose (glycogen) or storage lipids (triacylglycerols and other components of lipid droplets) (Figure 4). Nutrients can also be used in biosynthetic reactions, as for example glucose is converted in reactions of the pentose phosphate pathway to precursors of nucleic acids (Figure 4). Notably, this biochemistry converges on mitochondria, organelles that host the machinery for many metabolic processes (Figure 4).

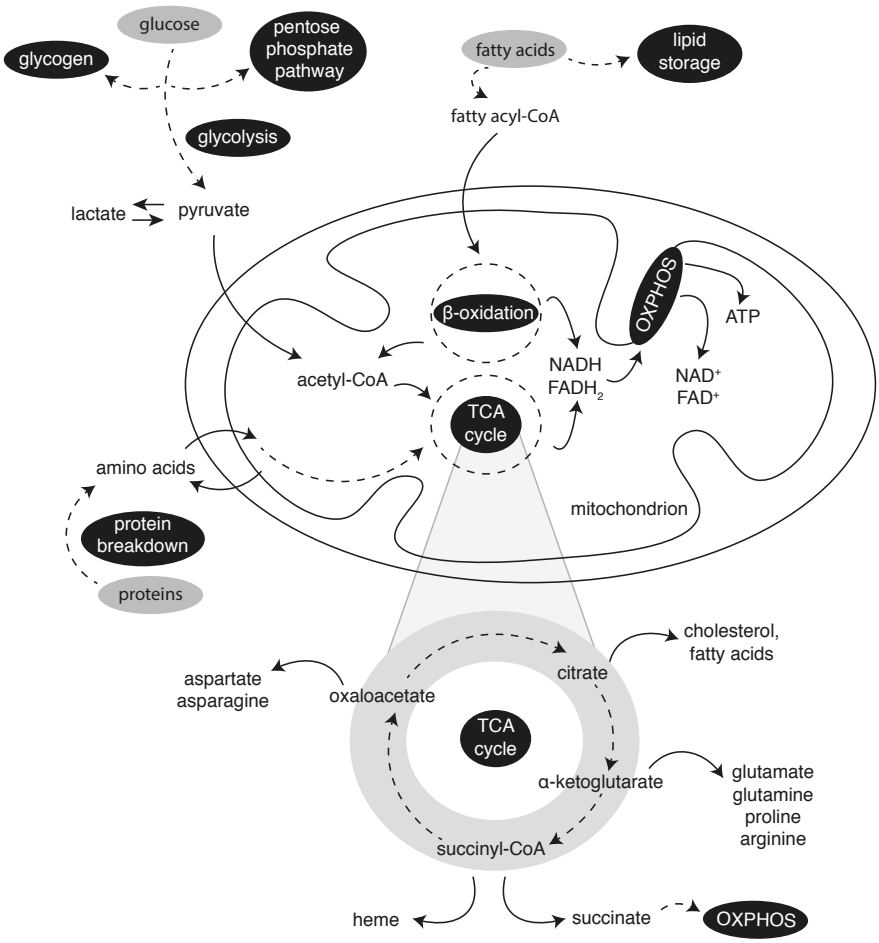

Figure 4. Simplified scheme of central metabolic pathways. Based on (Nelson, Lehninger, and Cox 2008). TCA = tricarboxylic cycle.

Metabolic pathways are not isolated entities, as the end and side products of a pathway may act as substrates for other metabolic pathways, forming immensely intricate metabolic networks. For example, products of the intermediate steps of the tricarboxylic cycle can 'leave' the cycle to serve as precursors for a plethora of other metabolites, notably amino acids and lipids (Figure 4). Likewise, products of amino acid breakdown can enter the tricarboxylic acid cycle (Figure 4). Many metabolic reactions are also reversible, where direction and relative flux depend on factors including the availability of substrates, enzymes, and cofactors.

The entire human or mouse metabolic network comprises thousands of reactions (Figure 5). Metabolic networks are however cell-specific, as only a fraction of these reactions takes place in a given cell type at a given time (Schultz and Qutub 2016; Bordbar et al. 2011; Minoru Kanehisa et al. 2010).

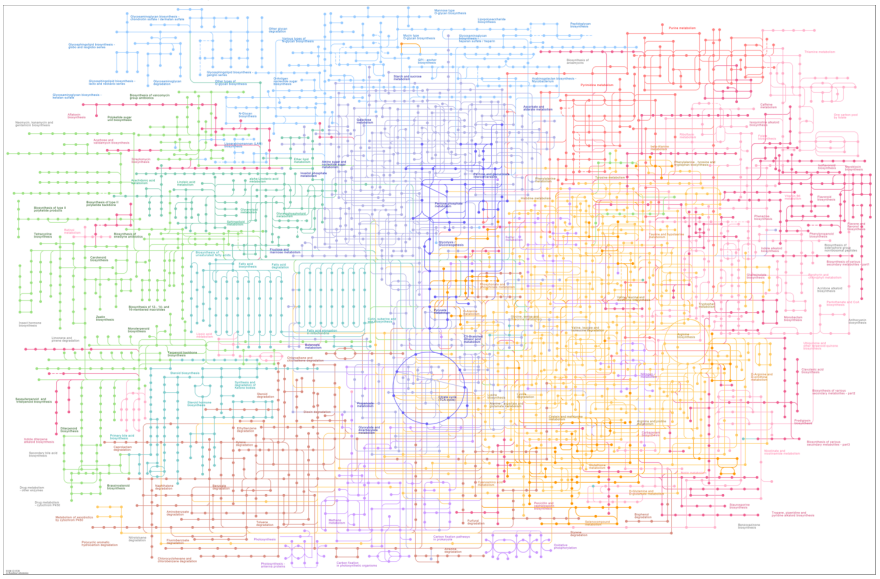

Figure 5. Reference metabolic pathway. Reproduced with copyright permission from Kyoto Encyclopedia of Genes and Genomes (map01100) (M. Kanehisa and Goto 2000; Minoru Kanehisa 2019; Minoru Kanehisa et al. 2020).

Intercellular metabolic cooperation takes place if the exported metabolites have signalling functions (for example, neurotransmitters), or are taken up by a different cell to be utilised within its metabolic network (for example, glucose uptake from blood) (reviewed in (Campbell et al. 2018)). Ideally, complete cell-specific metabolic networks and intercellular cooperation would be mapped directly from fate analyses of labelled metabolites in the brain. This is however limited by the sensitivity and resolution provided by existing techniques. To deduce indirectly, remarkably diverse cell biology and gene expression profiles may reflect the metabolic cooperation between brain cell types (Cahoy et al. 2008; Y. Zhang et al. 2014; Lewis et al. 2010; Eraso-Pichot et al. 2018). For example, amino acids act as building blocks of proteins, but in the brain these molecules can act as neurotransmitters and precursors of neurotransmitters. This function by definition involves cooperation between the cells. Another example is adenosine triphosphate (ATP): this molecule is most known as a chemical energy carrier, since hydrolysis of its phosphate groups leads to the release of energy that can be used in biochemical reactions. An equally important fate of this molecule however includes neurotransmission in the CNS. Enzymes involved in metabolism of amino and fatty acids are also differentially expressed in astrocytes compared to neurons, highlighting the possible cooperation between the cell types (Eraso-Pichot et al. 2018; Fecher et al. 2019; Russo et al. 2021).

Despite the limited ability to investigate cell-specific metabolic fluxes *in vivo*, the existing research has generated elegant hypotheses of trans-cellular metabolic pathways present within the brain. Some of these involve astrocyte-neuron cooperation and are introduced in greater detail below.

2.1.4.2 Examples of metabolic cooperation

Glutamate-glutamine shuttle. Glutamate is an amino acid that in the brain acts as the principal excitatory neurotransmitter. The glutamate-glutamine shuttle hypothesis suggests that neuronal activity is coupled to astrocytic uptake of glutamate. The mechanism would be as follows: i) glutamate is released by neurons to a synaptic cleft; ii) glutamate is taken up by astrocytes via astrocyte-enriched membrane transporters; iii) glutamate is converted to glutamine by astrocyte-enriched enzyme glutamine synthetase; iv) glutamine is released to the extracellular space by astrocytes; v) glutamine is taken up by neurons; vi) glutamine is converted to glutamate by neuron-enriched enzyme glutaminase, and re-used as a neurotransmitter (reviewed in (Stobart and Anderson 2013; McKenna and Ferreira 2016)). The view that astrocytes are a major contributor to glutamate uptake is consistent with the accumulation of extracellular glutamate when its uptake by astrocytes is disrupted, leading to neurotoxicity (reviewed in (Stobart and Anderson 2013; McKenna and Ferreira 2016)). However, the cell-specific enrichment of the aforementioned enzymes is not absolute. For example, oligodendrocyte lineage cells also express glutamate transporters and glutamine synthetase, contributing to the cycle (Xin et al. 2019). The shuttle is also not the only fate for glutamate, as it can also be converted to the inhibitory neurotransmitter **γ**-aminobutyric acid (reviewed in (McKenna 2007)), used as a building block for protein synthesis, provide an amino group for the biosynthesis of other amino acids, and also serve as a precursor for the tricarboxylic acid cycle.

Glucose-lactate shuttle. Glucose is the major blood-derived energy substrate for the brain. The glucose-lactate shuttle hypothesis postulates that astrocytic uptake of glucose from blood is coupled to neuronal activity as follows: i) neuronal activity stimulates glucose uptake by astrocytes from circulation; ii) glucose and glycogen are broken down to lactate in astrocytes; iii) lactate is exported by astrocytes; iv) lactate is taken up by neurons and used as an energy source (reviewed in (Pellerin and Magistretti 2012; Magistretti and Allaman 2015)). The hypothesis is conceptually compelling, as experimental evidence suggests that these aforementioned steps are biochemically possible, and astrocytic endfeet are positioned favorably for the nutrient uptake from blood (see 2.1.3.5). Neurons are however capable of independent uptake and metabolism of both glucose and lactate. Neuronal activity in these experiments is most often modelled through glutamate release. The investigated processes are influenced by the anesthesia *in vivo*, or in cultures by cell maturity and media composition. These factors possess a challenge to quantify the contribution of astrocytic activities to energy supply to neurons.

The directionality of metabolic fluxes and cell-specific contribution to net brain bioenergetics is a fundamental question to understanding brain biology, but due to limitations in existing research approaches is to date a subject of intense debate (reviewed in (Dienel 2017, 2012; Bak and Walls 2018)).

2.1.5 An ensemble

Consider the divergence of anatomical structures and regions within the mammalian brain: the cerebrum itself, the convoluted cortex with its layers, the olfactory bulb, the cerebellum, the hippocampus (Figure 6). Despite striking differences in regional anatomy, the cellular composition described in this chapter is shared across the entire brain. None of the cell types described above is ever found in isolation, surrounded by other cells of a kind. In fact, neurons, astrocytes, oligodendrocytes, oligodendrocyte progenitors cells, and microglia are invariably found in every part of the brain, tightly interconnected in a three-dimensional space, forming one of the most complex structures known to humankind.

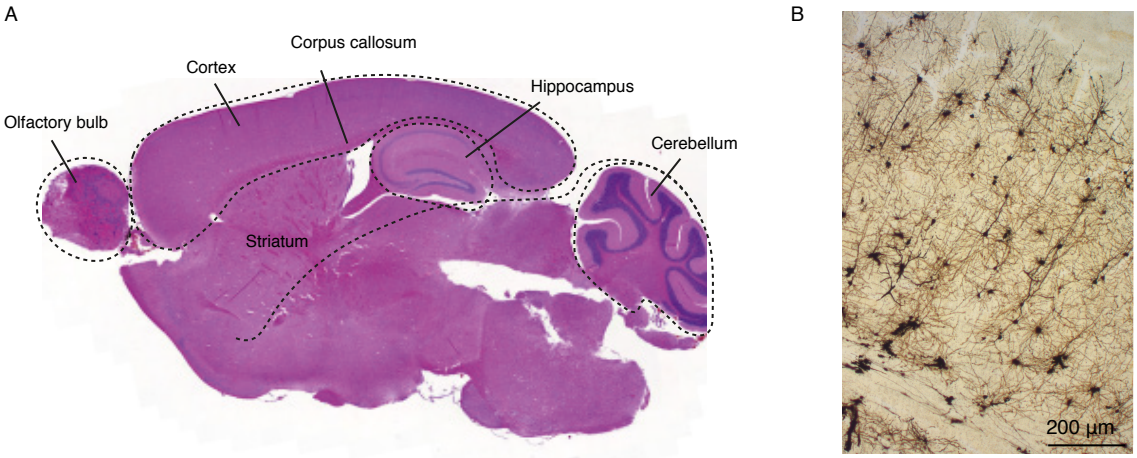

Figure 6. (A): Mouse brain, sagittal section. To demonstrate anatomical diversity, identifiable parts of major brain regions are indicated. Hematoxylin (blue, stains cell nuclei) and eosin (pink, stains cell cytoplasm and extracellular matrix) stain. (B): Mouse brain cortex, Golgi stain. Golgi stain by Babette Hollmann.

2.2 Cell biology of brain diseases

Brain disorders manifest with a variety of symptoms that affect consciousness, motor coordination and organ systems. They can range in severity from mild to lethal, in the age of onset from developmental pathologies to adulthood, and in duration from acute to chronic. In inherited and late-onset neurodegenerative brain diseases, the pathology is typically chronic. Brain cells in such diseases might display gradual, progressive changes over years or even decades. This contrasts conditions when the pathology is acute, such as traumatic brain injury, stroke, and infection. In those conditions, cell responses can be monumental and rapidly changing.

As the name suggests, neurodegenerative diseases manifest with degeneration of neurons (reviewed in (Przedborski et al. 2003)). Well-known examples of neurodegenerative diseases include Alzheimer's, Parkinson's, Huntington's diseases, and amyotrophic lateral sclerosis. That is not to say that the diseases are uniform, as the affected brain regions, neuronal subtypes, underlying molecular mechanisms, and clinical manifestations differ profoundly. The fact that neurodegeneration is a hallmark of many brain diseases suggests the vulnerability of neurons to various insults and changes in homeostasis. Nervous tissue has little capacity to regenerate neurons, making these diseases almost invariably progressive, presenting a great challenge for therapeutic interventions. Other constituent cell types of the brain are also affected in brain diseases. Degeneration of oligodendrocytes is also commonly observed, often resulting in pathologies of white matter (reviewed in (Ettle, Schlachetzki, and Winkler 2016; Ludwin 1997)). The degeneration of astrocytes and microglia, on the other hand, is rare, although for astrocytes is reported in some psychiatric diseases (reviewed in (Verkhratsky, Ho, et al. 2019)). As degeneration of neurons is evidently deleterious, and glial cells are often preserved in pathologies, views on pathogenesis became largely neurocentic (also discussed in 2.1.2). However, it is now recognised that all major brain cell types and their interactions are involved in activities indispensable for brain health (see 2.1). Understanding how the functions of all cell types are modified in pathologies is critical for development of treatments for brain diseases.

Across brain pathologies, astrocytes and microglia present with changes in morphology and function, collectively termed reactive gliosis (and distinguished as reactive astrogliosis and microgliosis). Conceptually, reactive gliosis could encompass the entire range of astrocytic or microglial responses in a pathological context. In practice, astrogliosis and microgliosis are most commonly assessed using histological readouts. Microglia typically display retraction or hypertrophy of cell processes, migration to the areas of degeneration, and sometimes proliferation (reviewed in (Streit, Walter, and Pennell 1999)). These phenotypes are scored using immunostainings against cytoplasmic markers, for example against IBA1 protein (ionized calcium-binding adapter molecule 1) (Figure 7A). These findings from published literature throughout the thesis are referred to as reactive microgliosis. Astrocytes in a diseased brain often display an increased expression of the cytoskeletal protein GFAP (Bignami et al. 1972; Eng et al. 1971; Smith, Somera, and Eng 1983). Typically, the number of detected $GFAP^+$ cells is increased, and the stained cell processes appear thicker and more numerous compared to a healthy setting (Figure 7B). This phenotype is often described as astrocyte hypertrophy, however, as discussed in 2.1.3.2, immunostaining against GFAP does not reveal the overall cell morphology. In fact, immunostainings against other astrocytic markers and dye-filling techniques showed that astrocyte number, general morphology, and

occupancy of non-overlapping domains can remain unchanged in pathology, despite the drastically different appearance of immunostaining against GFAP (Wilhelmsson et al. 2006). Notably, changes in the morphology of astrocytes and microglia often coincide, showing that both cell types are reactive to a wide range of insults and indicating a possible link between responses.

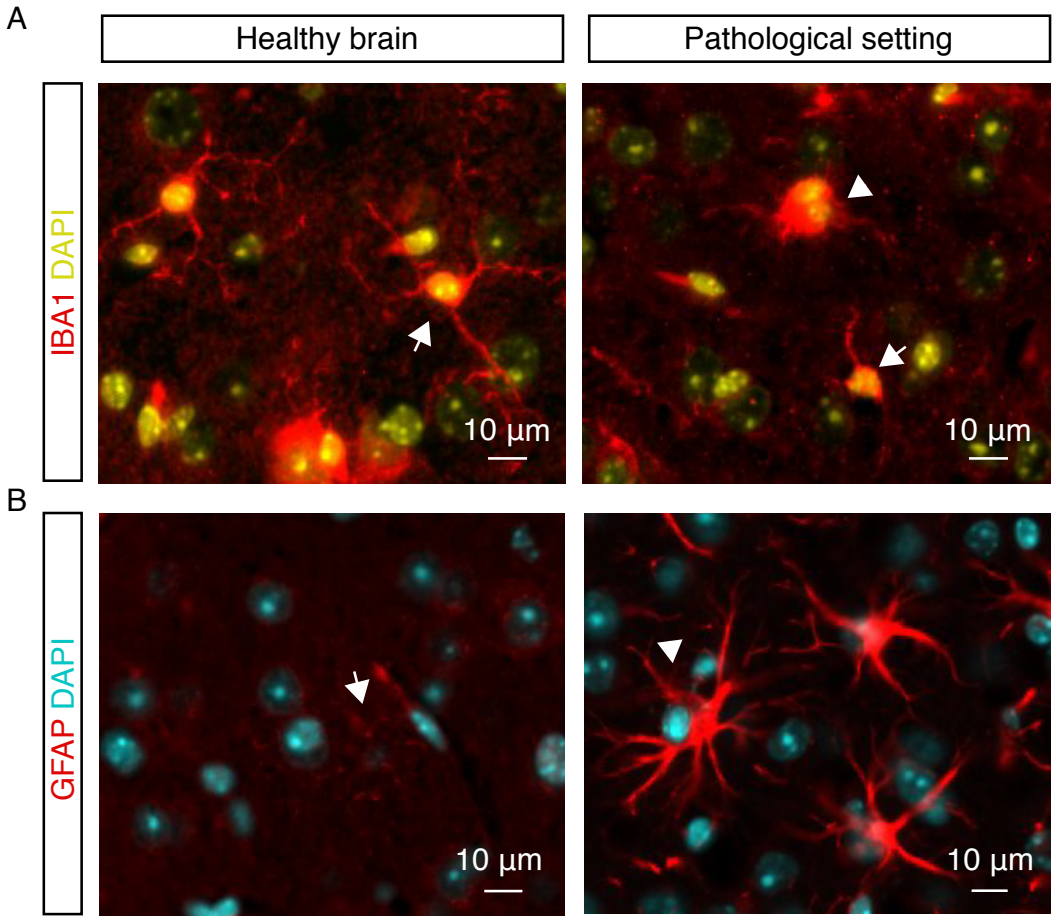

Figure 7. Immunofluorescence, mouse brain cortex. **(A):** Immunostaining against IBA1. Arrows indicate microglial cells with ramified morphology, an arrowhead indicates a microglial cell with ameboid morphology, typically defined as indicative of reactive microgliosis. **(B):** Immunostaining against GFAP. An arrow indicates weak GFAP signal, an arrowhead indicates a cell with upregulated GFAP expression, typically defined as indicative of reactive astrogliosis. Epifluorescence images.

In addition to morphological hallmarks, functions of astrocytes and microglia can be modified in a pathological context, and both cell populations display a wide range of changes in gene expression. Astrocytic responses are further discussed in 2.2.1. Changes in microglia often include modified expression patterns of surface molecules and released cytokines, resulting in either enhancement or attenuation of inflammatory responses (Streit, Walter, and Pennell 1999). Single-cell transcriptomic studies also revealed that these shifts are heterogeneous within the microglial population (Hammond et al. 2019; Olah et al. 2020). Elimination of microglia in healthy adult mice does not result in an evident pathology or

behavioral phenotypes (Elmore et al. 2014), but can have beneficial or detrimental consequences in disease (reviewed in (Green, Crapser, and Hohsfield 2020)).

To summarise, degeneration of neurons, and sometimes oligodendrocytes, are hallmarks of neurodegenerative diseases. Reactive astrogliosis and microgliosis occur ubiquitously across brain pathologies. Histological readouts are useful to detect cell responses, but have little capacity to inform on functional changes. The latter can be context-specific and contribute to either containment or aggravation of the pathology.

2.2.1 Astrocyte responses in brain pathologies

Astrocytes are involved in a plethora of activities that are essential for brain homeostasis (see 2.1.3). It would be logical to assume that these functions may be lost or modified in pathology. It would also be logical to assume that such changes can be context-specific, as an infection would affect astrocytes differently from a mechanical injury, a toxin, or a pathogenic variant perturbing, for instance, mitochondrial function. Examination of brain samples from patients with various chronic and acute pathologies however revealed that astrocytes consistently display an increased expression of intermediate filaments: notably GFAP, vimentin, and nestin (reviewed in (Sofroniew 2020; Liddelow and Barres 2017; Götz et al. 2015; Escartin et al. 2021)). However, the degree of this upregulation, the appearance of immunostainings, and the presence of cell proliferation differed between pathologies (reviewed in (Sofroniew and Vinters 2010; Milos Pekny and Pekna 2014)). Nevertheless, a ubiquitous upregulation of filament expression across brain pathologies directed the focus of the field for decades ahead. It has also been known that astrocytes can upregulate expression of various factors involved in immune responses, including cytokines, chemokines and antigen presentation molecules (Eddleston and Mucke 1993). Later research revealed that astrocytes display a wide range of context-dependent changes in gene expression and function in disease (Zamanian et al. 2012; Liddelow et al. 2017; Xinzhu Yu et al. 2020; Polyzos et al. 2019; L. E. Clarke et al. 2018; Boisvert et al. 2018; Anderson et al. 2016). It was then recognised that key to understanding disease mechanisms is a holistic investigation of astrocyte function. In this chapter, I review the directions of research that either focused on investigating the role of intermediate filament response, or how the overall function of astrocytes is modified in pathological conditions.

2.2.1.1 Intermediate filaments

First reported around 50 years ago (Bignami et al. 1972; Eng et al. 1971; Smith, Somera, and Eng 1983), the upregulation of GFAP expression in astrocytes became recognised as an ubiquitous hallmark of brain pathologies ranging from acute responses upon infections and trauma, to chronic neurodegenerative diseases (reviewed in (Sofroniew 2020; Liddelow and Barres 2017; Götz et al. 2015; Escartin et al. 2021)). The response appears to be ancient and evolutionarily conserved, as it also occurs in other mammalian (and at least some non-mammalian) species (reviewed in (Liddelow and Barres 2017; Ben Haim et al. 2015)). GFAP, vimentin, nestin are intermediate filaments expressed by radial glial cells during development (reviewed in (M. Pekny 2001)). In the adult CNS in the basal state, these proteins are expressed predominantly by astrocytes, ependymal cells and neural stem cells, but the expression in uninjured cortical astrocytes is low and may be undetectable. More recently, another intermediate filament, synemin, that is also normally expressed in the developing brain, was identified to increase in expression in astrocytes during pathology (Jing et al. 2007).The main

function of intermediate filaments is thought to be the contribution to cytoskeleton formation and therefore to provide structural support. These proteins may also participate in processes such as spatial coordination of proteasomes (Morrow et al. 2020), or mechanical protection of the nucleus during cell migration (Patteson et al. 2019). It is however not apparent how increased amounts of these proteins would affect astrocyte homeostasis and contribute to the pathogenesis of brain diseases.

The role of astrocytic intermediate filaments in homeostatic and pathological conditions was investigated by manipulating levels of their expression. Overexpression of human GFAP protein in mice resulted in accumulation of aggregates, and mice with high GFAP expression died within weeks after birth (A. Messing et al. 1998). In contrast, mice with genetic ablation of GFAP or vimentin were viable, generally healthy, and did not manifest with any evident brain pathology (Gomi et al. 1995; M. Pekny et al. 1995; McCall et al. 1996; Colucci-Guyon et al. 1994). Unexpectedly, there was no compensatory increase in expression of other intermediate filaments upon GFAP ablation (Gomi et al. 1995; M. Pekny et al. 1995; McCall et al. 1996). One study however reported abnormal myelination and hydrocephalus in GFAP-null mice, especially evident after 14 months of age (Liedtke et al. 1996). Together, these findings suggest that intermediate filaments may be dispensable for astrogenesis and astrocyte homeostasis, but their overexpression can nevertheless be harmful.

In a pathological context, mice deficient of GFAP displayed normal glial scarring after brain or spinal cord injury (M. Pekny et al. 1995, 1999) and scrapie infection (Gomi et al. 1995; Tatzelt et al. 1996). These mice were however more susceptible to injuries induced by free weight trauma (Nawashiro et al. 1998), and had a larger area of brain damage upon occlusion of middle cerebral and carotid arteries (Nawashiro et al. 2000). Mice devoid of both GFAP and vimentin proteins had dilated vessels in an uninjured state, and upon injury the scarring tissue was less dense, with increased number of fissures filled with blood and cellular debris (M. Pekny et al. 1999). Loss of GFAP or vimentin in mouse models of neurodegenerative diseases resulted in a slight shortening of lifespan of mice expressing a pathogenic variant of superoxide dismutase 1 (amyotrophic lateral sclerosis model) (Yoshii et al. 2011), and either in increase (Kraft et al. 2013) or no change (Kamphuis et al. 2015) in the amyloid load in mice expressing pathogenic variants of presenilin-1 and amyloid-beta precursor protein (Alzheimer's disease models).

An insight into consequences of GFAP dysregulation also comes from human genetics, as pathogenic variants in *GFAP* can cause Alexander disease (reviewed in (Albee Messing 2019)). This is a fatal neurological illness characterised by white matter degeneration, accumulation of Rosental fibers (GFAP-positive, dense osmiophilic structures) (Hsiao et al. 2005), and increased GFAP protein level (reviewed in (Albee Messing 2019)). Mouse models with knock-in pathogenic variants manifested with accumulation of Rosental fibers, but did not display myelination defects (Hagemann, Connor, and Messing 2006; Tanaka et al. 2007; L. Wang et al. 2015). In Drosophila and zebrafish, expression of pathogenic *GFAP* variants also induced the formation of aggregates (L. Wang, Colodner, and Feany 2011; S.-H. Lee et al. 2017). Mice that overexpress human GFAP and are knock-in for a pathogenic *GFAP* variant had a lifespan of less than 40 days (Hagemann, Connor, and Messing 2006). Two studies took an approach to differentiate astrocytes from induced pluripotent stem cells derived from patients with Alexander disease, comparing them with isogenic lines where a pathogenic variant was corrected (Li et al. 2018; Jones et al. 2018). Astrocytes with pathogenic variants displayed GFAP aggregates and had changes in gene expression (Li et al. 2018; Jones et al.

2018), negatively affected the number of oligodendrocyte progenitor cells and myelination in co-cultures (Li et al. 2018), had decreased propagation of calcium waves, and abnormal distribution of endoplasmic reticulum and lysosomes (Jones et al. 2018). Together, these studies suggest that pathogenic variants in *GFAP* have detrimental consequences for a cell, and lead to accumulation of GFAP aggregates. These phenotypes appear to be more similar to phenotypes of mice with GFAP overexpression compared to its loss.

In summary, this research has established an increased expression of intermediate filaments in astrocytes as a ubiquitous marker of a large variety of brain pathologies. Although this response might affect disease progression, it is likely that astrocyte homeostasis in pathologies may also be perturbed in other ways.

2.2.1.2 Context-dependent responses to pathology

Glial scar. In brain injury and stroke, astrocytes can become reactive and proliferative (Zamanian et al. 2012; Barreto et al. 2011; Cavanagh 1970), while astrocytes in mouse models of neurodegenerative diseases typically appear non-proliferative (reviewed in (Liddelow and Barres 2017; Ben Haim et al. 2015)). Both injury and stroke lead to cell death in the damaged area. Microglia and astrocytes around this area undergo massive functional and morphological changes and proliferate, together forming a barrier called glial scar (Schroeter et al. 1995). Cells from an injured area put in culture also gain the ability to form cellular aggregates (neurospheres) that can be differentiated into astrocytes, neurons, and oligodendrocytes (Buffo et al. 2008; Lang et al. 2004; R. Zhang et al. 2004). Developmental genes upregulated by reactive astrocytes include not only intermediate filaments, but also various factors involved in the regulation of cell cycle and neuronal differentiation (reviewed in (Götz et al. 2015; Escartin et al. 2021; Robel, Berninger, and Götz 2011)). In addition, reactive astrocytes can upregulate signalling pathways that are normally most active at developmental stages, including fibroblast growth factor, epidermal growth factor, vascular endothelial growth factor, sonic hedgehog, and Wnt signalling (reviewed in (Robel, Berninger, and Götz 2011)). Together, this suggests partial de-differentiation of astrocytes under certain pathological conditions.

It has been reasoned that glial scar formation prevents axonal regeneration, and therefore can impede tissue recovery. It has been also reasoned that such a barrier might limit inflammatory processes to a damaged area, preventing the spread of a pathology. Unlike asymptomatic ablation of microglia, ablation of astrocytes results in paralysis and death, making investigation of astrocyte contribution to pathology by cell ablation rather irrelevant (reviewed in (Jäkel and Dimou 2017)). Ablation of dividing cells would however test the role specifically of astrocyte proliferation and glial scarring. This became possible by targeting expression of thymidine kinase from the herpes simplex virus to astrocytes (Fischer et al. 2005). Administration of an antiviral agent ganciclovir to these mice results in disruption of DNA synthesis and apoptotic death of proliferating cells that express viral thymidine kinase, while non-proliferating cells remain unaffected (Fischer et al. 2005). Ablation of proliferating astrocytes in mice with stab cortical injury (Bush et al. 1999; Anderson et al. 2016) and with experimentally induced autoimmune encephalitis (mice are administered with myelin and viral or bacterial antigens) (Voskuhl et al. 2009) led to the worsening of mouse condition, severe inflammation, and increased tissue damage. In addition to acute glial scarring, it was tested whether ablation of chronic astrocytic scars upon spinal cord injury would promote neuronal regeneration (Anderson et al. 2016), but this also did not result in improvement of the phenotype. Another piece of evidence of a protective role of astrocyte responses upon injury

was the ablation of the transcription factor STAT3, which responds to inflammatory cues. This led to attenuation of astrogliosis and exacerbation of spinal cord injury (Herrmann et al. 2008; Okada et al. 2006; Anderson et al. 2016). Together, these studies demonstrated that glial scar can be beneficial for containment of pathology and recovery from injury.

Neuroinflammation. The pathogenesis of infections is variable, but commonly involves a rapid microglial response, followed by reactive astrogliosis. A widely used model to stimulate an infection-like response is intraperitoneal administration of lipopolysaccharide (a component of bacterial membrane), which induces a rapid systemic inflammatory response. In laboratory mice, lipopolysaccharide administration can result in extreme sickness behaviour, body weight loss, decreased activity or lethargy, and can be lethal (reviewed in (Lasselin et al. 2020)). Severe manifestations may therefore present a system more closely resembling a septic shock than a non-life-threatening infection. The severity of this response is dose-dependent, and possibly exacerbated when mice are housed in barrier facilities and have not previously encountered infections.

Peripheral administration of liposaccharide results in strong reactive microgliosis and astrogliosis (Buttini and Boddeke 1995; Herx and Yong 2001). This provides important evidence of astrocyte responses to inflammatory cues. Importantly, lipopolysaccharide has a very low to no ability to cross the blood-brain barrier. However, signalling within the nervous tissue may be induced by peripheral inflammation, by lipopolysaccharide molecules in brain vessels, or by a small number of molecules that penetrate the blood-brain barrier (Banks and Robinson 2010).

Astrocyte profiling. A more holistic understanding of how astrocytes respond to pathological contexts was derived from comparing transcriptomes of astrocytes sorted from mice injected with lipopolysaccharide or after transient middle cerebral artery occlusion (Zamanian et al. 2012). In both cases, astrocytes displayed an increased expression of intermediate filaments (Zamanian et al. 2012). The study focused on genes with upregulated expression, and transcriptome analysis using a microarray panel showed that over 50% of the genes induced by lipopolysaccharide were also induced by the middle cerebral artery occlusion (Zamanian et al. 2012). These included factors of the extracellular matrix, cell cycle genes (more prominent upon middle cerebral artery occlusion), immune response and cytokine signalling (more prominent upon lipopolysaccharide administration) (Zamanian et al. 2012). In both cases, the response was temporarily regulated, as in seven days the expression of many induced genes was normalised (Zamanian et al. 2012). Importantly, there were also genes upregulated only in one of the pathological contexts, providing the evidence of heterogeneity of reactive astrogliosis (Zamanian et al. 2012).

Based on this study, a set of gene expression markers was established: genes that define pan-reactive astrocytes, those specific to lipopolysaccharide administration (also called A1 astrocyte markers), and those specific to middle cerebral artery occlusion (also called A2 astrocyte markers) (Zamanian et al. 2012; Liddelow et al. 2017). Further research from the same laboratory identified that lipopolysaccharide does not evoke the same astrocytic response in mice that lack microglia or in purified astrocyte cultures (Liddelow et al. 2017). Instead, the response was mediated by microglia-secreted molecules, whereby in cultured cells necessary and sufficient for this induction was a cocktail of interleukin 1**α** (IL-1**α**), tumour necrosis factor (TNF) and complement component 1, subcomponent q (C1q) (Liddelow et al. 2017). Global IL-

1**α**$^{-/-}$TNF$^{-/-}$C1q$^{-/-}$ knockout mice also did not induce the set of lipopolysaccharide-specific markers of reactive gliosis(Liddelow et al. 2017). Together, this established a mechanism by which inflammatory cues evoke microglial response, in turn stimulating astrocytic response.

Furthermore, the functional consequences of this response were investigated. Astrocytes stimulated by IL-1**α**+TNF+C1q cocktail did not promote synapse formation when co-cultured with neurons, had diminished phagocytic activity, and the conditioned media was toxic for several neuronal subtypes and mature oligodendrocytes (Liddelow et al. 2017). This suggested that astrocytes lost homeostatic functions, and gained a neurotoxic function. To test the function of IL-1**α**+TNF+C1q-mediated response in neurodegenerative pathologies, the phenotypes of several mouse models were investigated in mice with IL-1**α**$^{-/-}$TNF$^{-/-}$C1q$^{-/-}$ global knockout: i) in mice with the optic nerve crush or microbeads injected to the eye (model of glaucoma), neuronal death was prevented (Liddelow et al. 2017; Guttenplan, Stafford, et al. 2020); ii) in transgenic mice expressing a pathogenic variant of human superoxide dismutase 1 (a model of amyotrophic lateral sclerosis), this led to remarkable lifespan extension and prevention of body weight loss, delayed neurodegeneration, and improved motor performance (Guttenplan, Weigel, et al. 2020); iii) after infection with prions, IL-1**α**$^{-/-}$TNF$^{-/-}$C1q$^{-/-}$ mice had an accelerated disease course and shortened lifespan (Hartmann et al. 2019). Finally, a drug that prevented release of these cytokines by microglia had a neuroprotective effect in mice injected with **α**-synuclein preformed fibrils, and in mice expressing human **α**-synuclein pathogenic variant (Parkinson's disease models) (Yun et al. 2018). Together, this research showed that the inflammatory IL-1**α**+TNF+C1q-mediated response can have context-dependent consequences important for disease progression.

These pioneering studies were followed by profiling of astrocyte responses in multiple disease models, and further investigated also at the single-cell level. Astrocyte responses appear to be both context-specific (Diaz-Castro et al. 2019; Xinzhu Yu et al. 2020; Anderson et al. 2016; L. E. Clarke et al. 2018; Boisvert et al. 2018) and heterogeneous at the cell population level (Al-Dalahmah et al. 2020; Mathys et al. 2019; Wheeler et al. 2020). A challenge for the field now is to elucidate the specificity and the functional outcomes of these responses.

2.2.1.3 Reactive astrogliosis

Astrocytic phenotypes in brain pathologies have been referred to with an array of terms: 'astrocytosis', 'astrogliosis', 'reactive gliosis', 'astrocyte activation', 'astrocyte reactivity', 'astrocyte re-activation', and 'astrocyte reaction' (from (Escartin et al. 2021)). To unify the terminology, a consensus statement, authored by over 80 researchers in the field, suggested the usage of terms 'reactive astrogliosis' and 'reactive astrocytes' (Escartin et al. 2021). By definition, reactive astrogliosis would be used to describe astrocyte responses to extrinsic factors. For pathologies that affect astrocytic cells, such as inherited pathogenic variants, the term 'diseased astrocytes' is suggested (Escartin et al. 2021). Strictly speaking, it however may be difficult to claim a pathology as exclusively extrinsic to astrocytes, except for models where only non-astrocytic cells are targeted (for example, neuron-specific conditional gene knockouts). It is also suggested the reactive gliosis would not be defined only by increased expression of GFAP, but an investigation of astrocytic functions would be carried out (Escartin et al. 2021). Assessment of GFAP expression indeed provides little information on astrocyte functions, but is however useful to investigate the presence of astrocytic responses in a condition of interest. Throughout this thesis, such findings from published literature are therefore referred to as reactive astrogliosis.

To define a reactive astrogliosis signature at the gene expression level, a set of gene expression markers was proposed (Escartin et al. 2021). The selection was based on studies that investigated various extrinsic and intrinsic factors related to astrocyte pathologies (Escartin et al. 2021). In a patahological context, the expression of genes defined as A1, A2, and pan-reactive astrogliosis markers (Liddelow et al. 2017; Zamanian et al. 2012), as well as of the genes from a recently suggested refined gene list (Escartin et al. 2021) can be altered. Thus, in the research presented in this thesis, both sets of markers were used to investigate the reactive astrogliosis signature (see 5.1.4, III).

In summary, it is now clear that astrocytes are essential for brain homeostasis and in pathologies undergo complex context-specific changes. Inquiries on contribution of astrocyte responses to pathogenesis is an active research area.

2.3 Mitochondria

One hallmark of eukaryotic cells is a network of continuously moving membranous structures: the mitochondria (Figure 8). At the end of the 19th century, Richard Altmann developed a new cellular staining, which allowed him to visualise and depict such networks (Altmann 1890). He hypothesized that these were autonomous intracellular organisms with metabolic and genetic functions, naming them 'life germs or bioblasts' (Altmann 1890). The term mitochondria from Greek 'mitos' (thread) and 'chondros' (granule) followed shortly after (Benda 1898). Within the following decades, the main characteristics of mitochondria were established: i) cellular respiration and key metabolic pathways take place in mitochondria (see 2.1.4.1, reviewed in (Ernster and Schatz 1981; Pagliarini and Rutter 2013)); ii) mitochondria incorporate amino acids into proteins (McLean et al. 1958); iii) mitochondria harbor DNA, which is replicated independently from nuclear DNA (Sherman et al. 1966; Nass and Nass 1963; Schatz, Haslbrunner, and Tuppy 1964). The true nature of these structures was a subject of debate for almost a century, but it is now accepted that mitochondria are eukaryotic organelles of bacterial origin.

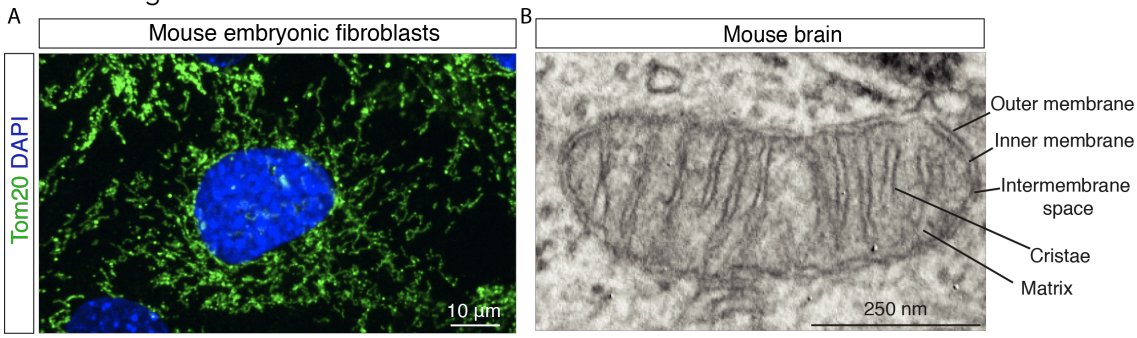

Figure 8. Mitochondria. **(A):** primary mouse embryonic fibroblast culture. Immunostaining against the protein of the outer mitochondrial membrane TOM20. **(B):** transmission electron microscopy, a mitochondrion in the mouse brain.

Mitochondrial morphology is determined by the cell type, metabolism, developmental programs and level of cell stress. Typically, mitochondrial networks form a tight perinuclear ring and are more diffuse in the cell soma (Figure 8). Mitochondria form contacts with other organelles, of which those with the endoplasmic reticulum are especially prominent (reviewed in (Rowland and Voeltz 2012)). Steady state morphology of the mitochondrial network is regulated by continuous fusion, fission, and motility events that are critical for cell homeostasis (Ishihara et al. 2009; Hsiuchen Chen et al. 2003). Additionally, mitochondria generate vesicles that deliver selective cargo to other organelles, namely to lysosomes and peroxisomes (Emélie Braschi et al. 2010; Neuspiel et al. 2008; Sugiura et al. 2014; Soubannier et al. 2012).

Mitochondria are enclosed by a compact outer membrane; the second membrane inside mitochondria forms convoluted invaginations known as cristae (Figure 8). This divides the mitochondrion to the intermembrane space and matrix, which compartmentalise distinct molecular machinery. Oxidative phosphorylation (OXPHOS) complexes span the cristae, while many metabolic reactions are located in the matrix. The difference of pH and voltage gradients between the intermembrane space and the matrix forms an electrochemical gradient named membrane potential. This charge is essential for the protein import in mitochondria, and therefore for all organellar functions. Both the mitochondrial network and the structural

organisation of mitochondrial compartments are highly divergent between cell types, but can also rearrange upon metabolic and other environmental cues (reviewed in (Pfanner, Warscheid, and Wiedemann 2019; Nunnari and Suomalainen 2012)).

2.3.1 Evolutionary origin

Mitochondria harbor a multi-copy genome, which is replicated within the organelle independently from nuclear genome replication. Phylogenetic analysis strongly supports the monophyletic origin of mitochondria from ancient **α**-proteobacteria ((Ku et al. 2015), reviewed in (Gray 2012)). There are no known eukaryotic lineages without mitochondria. This is supportive of a hypothesis that a single endosymbiotic event occurred between **α**-proteobacteria and another organism devoid of nuclei, likely archaea (reviewed in (Gray 2012)). The event is approximated to have occurred 1.4-2 billion years ago, governing the formation of the eukaryotic cell.

Protomitochondria likely harboured a large bacterial genome at a megabase scale, while modern mitochondrial genomes across the animal kingdom are much smaller, around 15-20 kb (reviewed in (Boore 1999)). This genome reduction likely occurred via a combination of the loss of genetic material and endosymbiotic gene transfer to the nucleus, thus establishing the transformation from an independent endosymbiont to a true cellular organelle. The majority of mitochondrial proteins are now encoded in the nucleus, synthesized in the cytoplasm, and imported in mitochondria (Andersson et al. 1998; Rath et al. 2020). Surprisingly, only 10-20% of mitochondrial proteins are putatively of **α**-proteobacterial origin, indicative of the expansion process (Gray 2015). The rest of mitochondrial proteins likely originated from the host itself and from several eukaryotic and prokaryotic lineages, with some sequences lacking evident homologs (Gray 2015). Specifically, the mitochondrial proteome of modern organisms shows a multi-lineage, mosaic origin (Gray 2015). For example, the mitochondrial replicative helicase Twinkle, as well as DNA and RNA polymerases, share their origin with T-odd bacteriophages ((Spelbrink et al. 2001), reviewed in (Shutt and Gray 2006)).

The most prominent features of mitochondria that highlight their bacterial origin are the shape and motility, the double membrane, the genome, and the machinery of gene expression. The budding of mitochondrial vesicles is likely another ancestral feature, reminiscent of vesicles shed by bacteria for communication within a colony and defense against pathogens (reviewed in (Gould, Garg, and Martin 2016; Emelie Braschi and McBride 2010; McBride 2018; Deatherage and Cookson 2012)). Together, this demonstrates the complex history of mitochondrial and eukaryotic cell evolution.

2.3.2 Mitochondrial functions

Some well-known functions of mitochondria include energy metabolism, calcium buffering, iron homeostasis, maintenance of redox balance, cell cycle regulation and death, as well as several activities involved in immune responses. Mitochondria are often referred to as the metabolic hubs of the cell, as these organelles host enzymes that perform varied biochemical reactions (reviewed in (Spinelli and Haigis 2018; Pfanner, Warscheid, and Wiedemann 2019)). Metabolic function controls the availability of substrates and cofactors for an innumerable number of cell processes. These include the final breakdown of nutrients using oxygen to generate ATP, coupled to the production of DNA, RNA, lipids and protein precursors (Figure 4). The function of ATP production is mainly powered by OXPHOS activity, which also

generates the membrane potential across its inner membrane that is essential for all organellar functions. An indispensable mitochondrial function is the assembly of iron-sulfur clusters (reviewed in (Lill and Mühlenhoff 2008; Stehling and Lill 2013)). These small prosthetic groups delocalise electron density over Fe and S atoms, mediating electron transfer. This is required for the function of an array of proteins, including those involved in oxidative phosphorylation, the tricarboxylic acid cycle, DNA synthesis and repair, and ribosome function (reviewed in (Lill and Mühlenhoff 2008)). Loss of iron-sulfur clusters is therefore incompatible with life. Some eukaryotic species harbor functionally reduced mitochondria-like organelles (mitosomes) that are devoid of most metabolic functions. The assembly of iron-sulfur clusters is nevertheless still performed in these organelles (reviewed in (Stehling and Lill 2013)).

Oxidative phosphorylation. OXPHOS is the process by which the oxidation of high energy electron carriers NADH and $FADH_2$ to reduced NAD^+ and FAD^+ results in the generation of an electrochemical gradient that enables ATP synthesis (Figure 9) (reviewed in (Nelson, Lehninger, and Cox 2008; Osellame, Blacker, and Duchen 2012)). Additionally, the activity of oxidative phosphorylation complexes generate highly reactive radicals (reactive oxygen species), which are important signalling molecules.

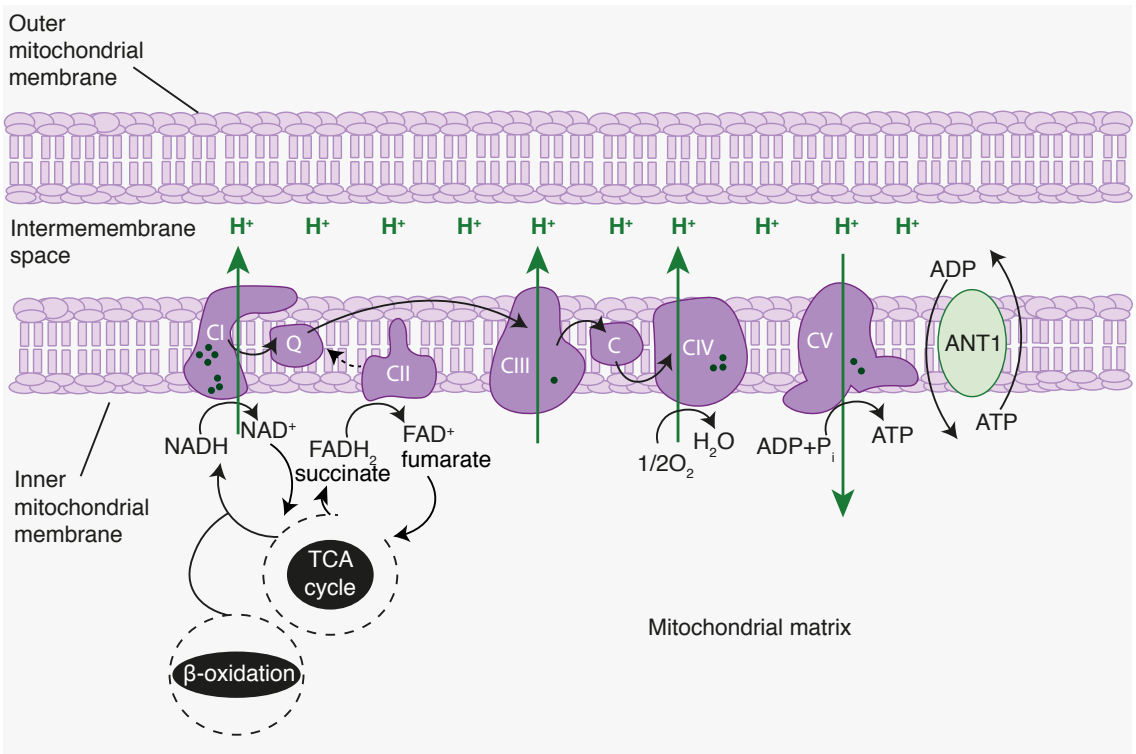

Figure 9. Schematic of oxidative phosphorylation (based on (Nelson, Lehninger, and Cox 2008; Suomalainen and Battersby 2018)). Green dots represent the number of protein subunits encoded in the mitochondrial genome (see 2.3.4). Arrows across the inner membrane indicate the direction of electron transport. CI-CV = Complex I - Complex V. Complex V = F_OF_1 ATP synthase. Q = coenzyme Q (ubiquinone). C = cytochrome c. ANT1 = ADP/ATP translocase 1. TCA = tricarboxylic acid.

As discussed in 2.1.4.1, major sources of NADH and $FADH_2$ are the tricarboxylic acid cycle and the **β**-oxidation pathways. Electron transfer is catalysed by several multisubunit protein complexes (Complex I-IV), and two soluble lipid carriers: coenzyme Q (also known as

ubiquinone or Q_{10}), and cytochrome complex (also known as cytochrome *c* or cyt *c*) (Figure 9). These components are localised in cristae invaginations of the inner mitochondrial membrane, and are commonly referred to as the 'electron transport chain'. Transfer of electrons from Complex I, III, and IV to the final electron acceptor oxygen, yields energy release, which fuels the pumping of protons from the matrix to the intermembrane space, resulting in an electrochemical gradient (Figure 9). Covalent attachment of ADP to inorganic phosphate (P_i) is catalysed by ATP synthase (F_OF_1 ATP synthase), also called Complex V (Figure 9). This requires passing protons through Complex V from the intermembrane space to the mitochondrial matrix using the proton gradient generated by the electron transport chain. Together, the electron transport chain and the ATP synthase form the oxidative ATP production unit, OXPHOS. ATP molecules are transferred across the mitochondrial membrane by the ATP-ADP carrier (adenine nucleotide carrier, ANT) (Figure 9). The flow of electrons is mediated by protein-bound redox cofactors, namely heme, flavin, copper, and iron-sulfur cofactors. An assembly of these prosthetic groups is thus essential for OXPHOS function.

Complex I and Complex II accept electrons through oxidation of NADH and $FADH_2$, the major sources of which are the breakdown of nutrients and macromolecules. Complex I (NADH:ubiquinone oxidoreductase) is a large protein complex comprising 45 subunits. Through the matrix-facing hydrophilic part of Complex I, NADH enters the inner channel of the complex, where it is oxidized to NAD^+. Complex II (succinate dehydrogenase, or succinate-coenzyme Q reductase) is an enzyme of the tricarboxylic acid cycle that catalyses the conversion of succinate to fumarate, resulting in oxidation of $FADH_2$ to FAD^+. This reaction serves as a second entry point to the electron transport chain. Oxidation of NADH and $FADH_2$ by these complexes restores the levels of NAD^+ and FAD^+, which can then re-enter the tricarboxylic acid cycle.

Electrons from both Complex I and Complex II are transferred to the lipid-soluble coenzyme Q, reducing it to ubiquinol. Ubiquinol diffuses within the membrane to transfer electrons to Complex III (coenzyme Q : cytochrome c – oxidoreductase). Complex III passes electrons to another lipid-soluble electron carrier, cytochrome *c*. Complex IV (cytochrome *c* oxidase) accepts electrons from cytochrome *c* and transfers electrons to the final electron acceptor, oxygen. The electron energy is gradually decreased during passage through the electron transport chain, and this energy is used to pump protons to the intermembrane space (Figure 9). Complex V utilises the electrochemical gradient to catalyze the reaction of ATP formation. The function of the electron transport chain can also be uncoupled from ATP synthase via action of several proteins that dissipate the electrochemical gradient. These proteins transport protons out of the intermembrane space, dissipating their energy as heat.

Complex V (F_OF_1 ATP synthase) comprises a water-soluble F_1, and the F_O membrane-embedded ring domains. Protons move down the electrochemical gradient through the F_O unit, energizing a conformational rotation of the F_1 unit. This catalyses the reaction of ADP and P_i binding, resulting in ATP synthesis. Complex V can also act in reverse and hydrolyse ATP, resulting in proton pumping into the intermembrane space and generation of membrane potential.

2.3.3 Specialisation of mitochondrial function in brain cell types

Mitochondria in different tissues and cell types can have different protein compositions and be involved in distinct activities. An early known example of such specialisation is the activity of a liver-enriched mitochondrial enzyme carbamoyl phosphate synthetase I (S. Clarke 1976; Lusty

1978). This is the rate-limiting enzyme in the urea cycle, a major pathway for ammonia detoxification.

Analysis of transcriptomes of individual cell types in the brain (Cahoy et al. 2008; Lovatt et al. 2007; Y. Zhang et al. 2014; Doyle et al. 2008) and of the mitochondrial proteome in mouse tissues (Pagliarini et al. 2008) allowed the comparison of the cell-specific expression of mitochondrial genes and proteins (Eraso-Pichot et al. 2018). This analysis revealed that genes involved in **β**-oxidation and amino acid metabolism are enriched in astrocytes compared to neurons (Eraso-Pichot et al. 2018). These findings were consistent with the mitochondrial proteome of cells purified from the adult mouse brain, as well as cultured cells derived from neonatal mice (Fecher et al. 2019; Russo et al. 2021). Biochemically, it was confirmed that astrocytes, but not neurons, are capable of performing **β**-oxidation (Eraso-Pichot et al. 2018; Fecher et al. 2019; Ioannou et al. 2019).

Together, these studies discovered cell-specific mitochondrial functions in astrocytes and neurons. This provided valuable insights for understanding the compartmentalisation of metabolic pathways within brain cell types, taking forward our understanding of cell-specific metabolic networks in the brain (see 2.1.4).

2.3.4 Genome(s) expression in mitochondrial function

The mitochondrial proteome comprises over a thousand proteins (Rath et al. 2020), of which only 1% are encoded by the mitochondrial genome; the rest are encoded by the nuclear genome (Figure 10). Nuclear-encoded mitochondrial proteins participate in all mitochondrial activities. The mitochondrial genome encodes several subunits of the oxidative phosphorylation complexes, as well as tRNAs and rRNAs for organellar gene expression (Figures 9-10). To function, the organelle requires coordinated expression of both the nuclear and mitochondrial genomes.

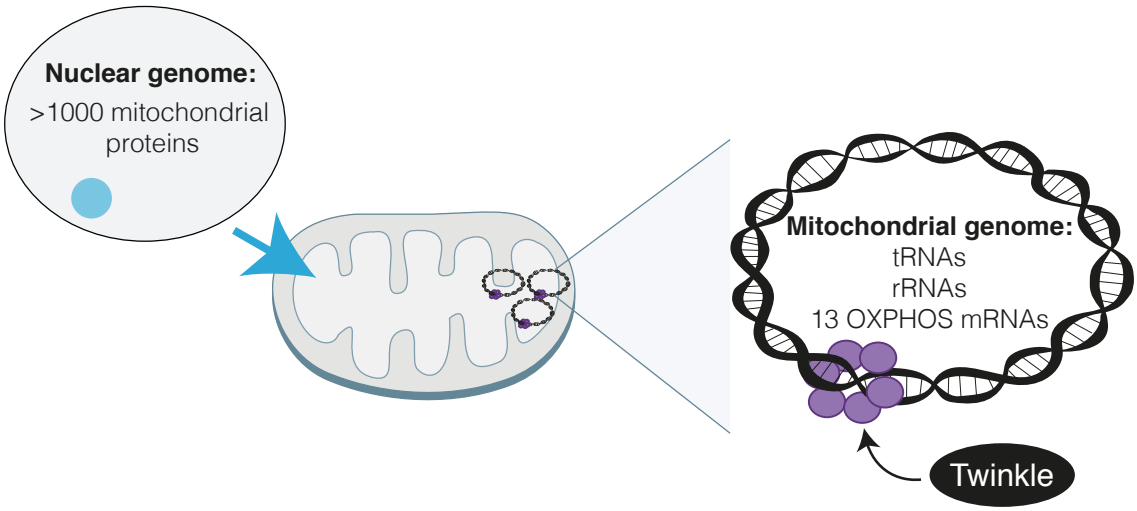

Figure 10. Schematic. Mitochondrial proteome requires coordinated expression of nuclear and mitochondrial genomes.

Oxidative phosphorylation. OXPHOS complexes consist of approximately 80 protein subunits, of which 13 are encoded in the mitochondrial genome (Figures 9-10). In addition to the remaining subunits, the nuclear genome encodes dozens of factors necessary for OXPHOS complex assembly (reviewed in (Diaz et al. 2011)).

Protein import. Nuclear transcripts are translated on cytoplasmic ribosomes, and are imported to mitochondria as unfolded precursors via import complexes residing in mitochondrial membranes (reviewed in (Schmidt, Pfanner, and Meisinger 2010)). Protein import through the inner mitochondrial membrane strictly depends on the membrane potential, and the latter is therefore essential for organellar function (reviewed in (Geissler et al. 2000)).

Mitochondrial inner membrane. OXPHOS complexes are integrated in the inner mitochondrial membrane. Mitochondrially-encoded proteins are extremely hydrophobic and inserted into the membrane co-translationally (Itoh et al. 2021). These co-translationally inserted subunits need to integrate in multi-subunit OXPHOS complexes. Discordance of protein translation disrupts the inner membrane and cristae organisation, creating membrane stress with broad consequences for organellar and cell homeostasis (reviewed in (Suomalainen and Battersby 2018; Battersby and Richter 2013)).

Mitochondrial genome expression. The mitochondrial genome is a multi-copy circular DNA molecule, the copy number of which depends on the cell type and stage of differentiation (reviewed in (Gustafsson, Falkenberg, and Larsson 2016)). Mitochondrial genome replication occurs in both dividing and postmitotic cells. Most components required for gene expression and the entire mitochondrial replication machinery are encoded in the nuclear genome (reviewed in (Gustafsson, Falkenberg, and Larsson 2016; Suomalainen and Battersby 2018)). The minimal mitochondrial replisome components are encoded in the nucleus: DNA polymerase gamma (POLG); helicase Twinkle; and the single-strand DNA-binding protein (mtSSB) (Korhonen et al. 2004). The mitochondrial transcription factor A (TFAM) can bind, unwind and bend DNA, acting as a DNA packaging factor (Kaufman et al. 2007). TFAM binding also appears to be important for mitochondrial transcription (reviewed in (D. Kang, Kim, and Hamasaki 2007). Physiological regulation of mitochondrial DNA (mtDNA) replication is poorly understood, however manipulation of Twinkle or TFAM levels proportionally affect the copy number of the mitochondrial genome (Tyynismaa et al. 2004; Ekstrand et al. 2004; Ylikallio et al. 2010; Milenkovic et al. 2013).

To summarise, mitochondrial function requires coordinated expression of the nuclear and mitochondrial genome. This is indispensable for the assembly of oxidative phosphorylation complexes, cristae organisation, and maintenance of the membrane potential for protein import to the organelle. Loss of mitochondrial gene expression primarily leads to failures in assembly of OXPHOS complexes, which has pleiotropic effects on organellar function and cell homeostasis (further discussed in 2.3.6).

2.3.5 Mitochondrial turnover

Pulse-chase labeling experiments invariably demonstrated that mitochondrial proteins, lipids and nucleic acids, like other biological molecules, have an inherent half-life (reviewed in (Poovathingal et al. 2012)). The turnover of mitochondrial components could in principle occur at the level of an entire network, its parts (e.g., 'an individual mitochondrion'), and individual molecules (e.g., individual proteins) (Figure 10).

MtDNA half-life differs between tissues, and in the brain is around 30 days, with cell-specific turnover rates currently unknown (Gross, Getz, and Rabinowitz 1969). Factors that degrade linearised fragments of mtDNA include proteins of the replication machinery and a mitochondrial exonuclease MGME1 (Peeva et al. 2018; Torregrosa-Muñumer et al. 2019). POLG activity is also required for the elimination of paternal mtDNA at least in Drosophila (Peeva et al. 2018; Torregrosa-Muñumer et al. 2019; Nissanka et al. 2018; Z. Yu et al. 2017). DNA turnover is also compatible with lysosomal turnover of mitochondrial cargo that is discussed below.

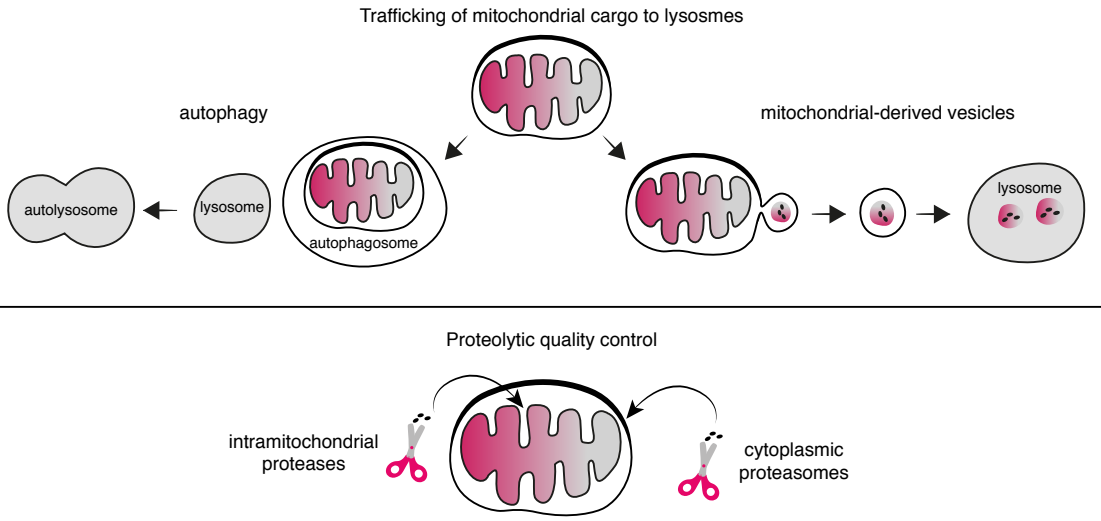

Figure 11. Schematic of the mechanisms of mitochondrial turnover.

Network level. In the majority of animals, including humans and mice, mitochondria are inherited maternally from the oocyte, and the network is partitioned at each cell division (reviewed in (Ladoukakis and Zouros 2017)). The organelles cannot be generated *de novo* and every nucleated cell contains mitochondria. Elimination of a mitochondrial network however can occur upon fertilisation (paternal mitochondria, (reviewed in (Cummins 2000)), in mammals upon maturation of erythrocytes (Sandoval et al. 2008; Schweers et al. 2007), and when severe damage to mitochondria occurs (for example, membrane depolarisation (Narendra et al. 2008). Since the elimination of a mitochondrial network otherwise results in cell death, it is reasoned that basal turnover occurs by other mechanisms.

Parts of the network. Parts of the mitochondrial network can be targeted to lysosomes. Two main mechanisms of trafficking of mitochondrial components to lysosomes were established: formation of mitochondrial-derived vesicles and autophagy (Figure 11). The latter can occur either via non-selective turnover (general autophagy), or via an enzymatic labelling of organelles for degradation (selective autophagy, or mitophagy). Several groups have developed assays using fluorescent proteins targeted to mitochondria that change their

emission spectrum in an acidic lysosomal environment (Sun et al. 2015; McWilliams et al. 2016; J. J. Lee et al. 2018; Katayama et al. 2020). This research invariably reported that a fraction of mitochondrially-targeted constructs ends up in lysosomes *in vivo*. The studies refer to these findings as mitophagy, however it is now established that observed lysosomal targeting of mitochondrial constructs is also compatible with mitochondrially-derived vesicles, a process regulated independently of autophagy and occurring through a distinct mechanism (Figure 11) (Soubannier et al. 2012; Sugiura et al. 2014; McLelland et al. 2014).

The current knowledge on the relative contributions of mitophagy, general autophagy, and mitochondrial-derived vesicles for the delivery of mitochondrial cargo to lysosomes is very limited. In a healthy adult mouse heart, serial electron microscopy revealed mitochondrially-derived vesicles, but no autophagosomes containing mitochondria (Cadete et al. 2016). Whereas, pharmacologically induced mitochondrial stress resulted in an increased number of mitochondrial-derived vesicles, peri mitochondrial vesicular clusters and autophagosomes containing mitochondria (Cadete et al. 2016). Generally, activity of both pathways is likely dependent on the developmental stage, the cell type, and can be affected by particular experimental settings, such as induced mitochondrial dysfunction. To make a distinction between the mechanisms is conceptually important, since pathways are independently regulated and have different biosynthetic requirements, such as the generation of an autophagic membrane. Additionally, mitochondrial-derived vesicles contain selective protein cargo (Neuspiel et al. 2008; Soubannier et al. 2012), while autophagosomes are considered to harbor all mitochondrial components.

Proteins. Turnover rates of mitochondrial proteins differ by orders of magnitude and display tissue-specific patterns, which are modified by nutrient availability (T.-Y. Kim et al. 2012; Jaleel et al. 2008; Vincow et al. 2013; Price et al. 2010; Karunadharma et al. 2015). This illustrates a major contribution of the turnover at the level of individual mitochondrial proteins. Such turnover is compatible with the action of numerous known mitochondrial proteases (reviewed in (Deshwal, Fiedler, and Langer 2020)), shuttling of selective cargo to mitochondrial-derived vesicles (Neuspiel et al. 2008; Soubannier et al. 2012), and the action of cytoplasmic proteasomes (Azzu and Brand 2010; Kowalski et al. 2018; Chan et al. 2011; Mårtensson et al. 2019). The activity of intramitochondrial proteases is established as essential for organellar homeostasis and cell fitness, the disruption of which can cause human disease (reviewed in (Deshwal, Fiedler, and Langer 2020)).

In summary, several mechanisms of mitochondrial component turnover have been established. Although their context-specific regulation and relative contributions remain elusive, where turnover of the mitochondrial genome is particularly poorly understood. Considering the importance of mitochondrial function, elucidation of mechanisms for organellar quality control is likely to illuminate key aspects of human health and disease.

2.3.6 Mitochondrial dysfunction

Disruptions in mitochondrial function result in broad consequences for cell homeostasis and fitness. The initial research concentrated on bioenergetic implications of mitochondrial dysfunction, namely defects in ATP production. The pleiotropy of phenotypes resulting from mitochondrial dysfunction is however challenging to explain by an ATP production deficiency alone. Here I focus on discussing the consequences of disrupted mitochondrial genome expression and OXPHOS function.

2.3.6.1 Mitochondrial diseases

Pathogenic mutations that disrupt mitochondrial function manifest as a heterogeneous group of diseases, formally united by the term primary mitochondrial diseases. Pathogenic variants can occur in the mitochondrial genome or nuclear genes that encode mitochondrial proteins (Rath et al. 2020). To date, pathogenic variants in more than 300 genes encoding mitochondrial proteins were identified as causative of human diseases (reviewed in (Koopman, Willems, and Smeitink 2012; Thompson et al. 2020; Suomalainen and Battersby 2018)), and the combined prevalence of these rare genetic diseases is estimated as 1 in 5,000 individuals (Skladal, Halliday, and Thorburn 2003; Gorman et al. 2015). Mitochondrial diseases are often severe and mostly lack curative treatments.

Defects in oxidative phosphorylation occur in almost every mitochondrial disease. This pathway is however primarily affected by pathogenic variants in subunits or assembly factors of the OXPHOS complexes, or in mtDNA and proteins regulating its replication and expression. Diseases in this group are extremely variable in clinical representation, can manifest at any age (neonatal manifestations are the most common and most severe), and be systemic or tissue-specific. The latter include encephalopathies, myopathies, cardiomyopathies, and hepatopathies (Carlo Viscomi, Ardissone, and Zeviani 2016; Suomalainen and Battersby 2018; DiMauro et al. 2013). An example of this phenotypic variability is *POLG*-related diseases that include a primary muscle-related disease progressive external ophthalmoplegia, as well as Alper-Huttenlocher syndrome (characterised by a clinical triad of severe epilepsy, liver degeneration and progressive developmental regression) (reviewed in (Saneto 2018; Sofou et al. 2012; Van Goethem et al. 2004)). Also, the age of onset of *POLG*-related mitochondrial recessive ataxia syndrome ranges from early childhood to several decades into adulthood (Hakonen et al. 2005; Rantamaki et al. 2001; Hakonen et al. 2008). The reasons underlying the range of clinical manifestations are poorly understood, but are unlikely to be explained by the biochemical defect in the ATP synthesis alone.

Leigh syndrome is a prime example of a mitochondrial disease stemming from OXPHOS defects (Leigh 1951). Classically, the disease manifests before two years of age with vomiting and diarrhea, muscle weakness and involuntary contractions; the spectrum can also include epilepsy and respiratory deficiency (reviewed in (Baldo and Vilarinho 2020; Chang et al. 2020)). Pathogenic variants in at least 75 genes in both mitochondrial and nuclear genomes are currently identified as causing Leigh disease (reviewed in (Lake et al. 2016)). These include variants leading to dysfunction of all OXPHOS complexes and disruption of coenzyme Q metabolism (reviewed in (Baertling et al. 2014)). Manifestation, severity and prognosis vary, and can be partially inferred from an exact genetic diagnosis (Lake et al. 2016). The disease is invariably progressive and often leads to lethality. Neuroimaging hallmarks of Leigh disease include symmetric brain necrotic lesions where basal ganglia, cerebellum and brainstem are commonly affected. Leigh's disease was modeled by inducing in mice a knockout of *Ndufs4*, which encodes a subunit of Complex I. The model became useful to discover several treatment strategies, some of which might also be applicable to other mitochondrial diseases (further discussed in 2.3.6.4).

Mitochondrial encephalopathies vary in histopathological manifestations and brain regions affected. Most commonly, clinical findings include neurodegeneration and reactive gliosis (typically assessed by immunostainings against GFAP and IBA1) (reviewed in (Lake et al. 2015; Brown and Squier 1996)). Myelin abnormalities also occur in a number of mitochondrial

diseases (reviewed in (Bindu et al. 2018)). Although a pathogenic variant may affect function of each cell type of a patient, neurons are thus far considered to be the main disease-driving cell type.

Mitochondrial dysfunction is a hallmark of neurodegenerative diseases, such as Alzheimer's, Huntington's and Parkinson's diseases, multiple sclerosis, and amyotrophic lateral sclerosis (reviewed in (Palomo and Manfredi 2015; Lin and Beal 2006; Y. Wang et al. 2019). Inferring the cause-consequence of secondary mitochondrial dysfunction is a challenge, and better understanding of primary mitochondrial diseases is likely to strengthen our understanding of common neurodegenerative diseases (reviewed in (Murphy and Hartley 2018)).

2.3.6.2 Mouse models of mitochondrial diseases

Animal models of human diseases are typically designed to have an inborn or an induced pathology that is equivalent to the disease. Ideally, an animal model would recapitulate the original cause and manifestation of the disease, and have a strong likelihood to predict treatment outcomes (reviewed in (Ericsson, Crim, and Franklin 2013; Rosenthal and Brown 2007)). Although models that closely correspond to these criteria are rather rare, animal models have proven to be useful to investigate human diseases. Mouse models are commonly used due to the relatively high genetic similarity to humans, small size, ease of handling, and tools available to manipulate the genome.

Mitochondrial diseases caused by pathogenic variants in the nuclear genome provide a blueprint to design mouse models that recapitulate the original cause of human disease. Since seemingly the first report in 1995 (Y. Li et al. 1995), functions of approximately 50 mitochondrial proteins were targeted in mice (reviewed (Ruzzenente, Rötig, and Metodiev 2016; Torraco et al. 2015; Iommarini et al. 2015; Peralta et al. 2015)). Common approaches include constitutive or conditional gene inactivation (Cre-Lox recombination system is discussed in 2.4) and expression of a pathogenic variant that either substitutes or is expressed in addition to a wild type allele.

Complete loss of function of mitochondrial proteins is often embryonic lethal, and models with patient-mimicking pathogenic variants would in principle provide a more accurate recapitulation of a disease cause. However, an analogous patient pathogenic variant does not necessarily cause a relevant phenotype in mice, and combined usage of different models may be beneficial. For example, pathogenic variants in *TWNK* manifest as a variety of human diseases, including autosomal dominant progressive external ophthalmoplegia. Germline knockout of mitochondrial replisome genes including *Twnk* is embryonic lethal (Larsson et al. 1998; Hance, Ekstrand, and Trifunovic 2005; Milenkovic et al. 2013). Mice that express patient-mimicking the dup353-365 disease variant in *Twnk* (Deletor mice) recapitulate several molecular hallmarks of the human disease, namely mtDNA deletions, occurrence of muscle fibers with complex-specific OXPHOS deficiency, and gene expression responses (Tyynismaa et al. 2005, 2010; Nikkanen et al. 2016; Forsström et al. 2019). This enabled the use of Deletor mice in several preclinical trials where the effectiveness of treatments to these molecular hallmarks were scored (N. A. Khan et al. 2017, 2014; Ahola-Erkkilä et al. 2010; Nikkanen et al. 2016). Two of these trials were also further extended to human patients (Pirinen et al. 2020; Ahola et al. 2016). While useful to investigate molecular pathogenesis, Deletor mice however do not display any motor defects, imposing a challenge to draw definitive conclusions on the impact of interventions on morbidity. In comparison, conditional *Twnk* knockout in the heart

and muscle causes OXPHOS dysfunction in these tissues, signs of cardiomyopathy, and leads to shortened mouse lifespan (Milenkovic et al. 2013; Kühl et al. 2017). This model does not directly correspond to a cause of human disease, but could be leveraged to test interventions aimed at compensating for consequences of a severe mitochondrial dysfunction. A limitation of conditional knockout of essential mitochondrial genes is that often it results in cell death, which does not always recapitulate human pathogenesis.

Examples of successful recapitulation of a mitochondrial disease phenotype in mouse models include a complete loss of protein function that mimic a similar defect in patients. Of these, loss of *Ndufs4* and *Coq9* are discussed below. Loss of the Complex I subunit NDUFS4 is one of the most widely used mouse models of a mitochondrial disease. Mice with constitutive or neuroglial (driven by Nestin-Cre) NDUFS4 deficiency manifested with growth retardation, but appeared healthy until ~5 weeks, when they developed ataxia and died at the age of ~7 weeks (Kruse et al. 2008). The viability of these mice at post developmental stages is likely explained by the fact that the Complex I is partially assembled and has residual activity in the absence of NDUFS4 (Stroud et al. 2016; Kruse et al. 2008). These mice with NDUFS4 loss are considered as a Leigh disease model (Leigh disease is introduced in 2.3.6.1). Disrupted metabolism of coenzyme Q in humans manifests as a variety of diseases that include encephalopathy, myopathy, nephropathy, and multisystem disorders (Emmanuele et al. 2012). In mice, the disease was modelled by expressing a pathogenic variant in *Coq9* gene, that leads to the deficiency of COQ7 protein function (García-Corzo et al. 2013). This resulted in a growth retardation, progressive loss of locomotor function, and premature lethality at the age of 3-5 months (García-Corzo et al. 2013). Histopathological characterisation of both *Ndufs4* knockout and *Coq9* mutant mice showed neurodegeneration and vacuolation of brain parenchyma, accompanied by reactive astrogliosis and microgliosis (assessed by immunostaining against GFAP and IBA1) (Quintana et al. 2010; García-Corzo et al. 2013). The affected brain regions however differed in mice with *Ndufs4* and *Coq9* knockout, and vulnerability of individual neuronal subtypes or the cellular basis of spongiotic degeneration were not investigated, making it challenging to infer possible similarities between the models.

Loss of a protein function in mice can also lead to disparate consequences compared to human diseases. An example of this is a nucleotide salvage enzyme thymidine kinase 2. Deficiency of thymidine kinase 2 in humans primarily results in myopathy or a disease similar to spinal muscular atrophy (Mancuso et al. 2003; Saada et al. 2001; Oskoui et al. 2006). Mice deficient for the same enzyme manifested with severe growth retardation, early lethality and brain pathology, but skeletal muscle was relatively unaffected (Akman et al. 2008; Zhou et al. 2008).

Many mitochondrial functions are conserved in evolution. Mouse models of mitochondrial diseases have proven to be useful, but also have limitations which may result in imperfect predictive power. Different approaches in model design may reflect distinct aspects of human diseases. Whether the specific findings in a model are applicable to other models and human patients would require specific studies.

2.3.6.3 Cell responses to mitochondrial dysfunction

Mitochondrial dysfunction induces a range of cell responses that might affect the pathological manifestations. These include activation of the integrated stress response (ISR) (Figure 12). ISR is an evolutionary conserved signalling pathway that acts to attenuate cytoplasmic protein synthesis and to simultaneously evoke the action of a set of transcription factors (TFs), leading to remodelling of the biosynthetic programs (Figure 12). ISR is triggered by a range of cell intrinsic and extrinsic insults including nutrient deficiency, infections, hypoxia, and unfolded protein stress (Figure 12) (reviewed in (Pakos-Zebrucka et al. 2016)).

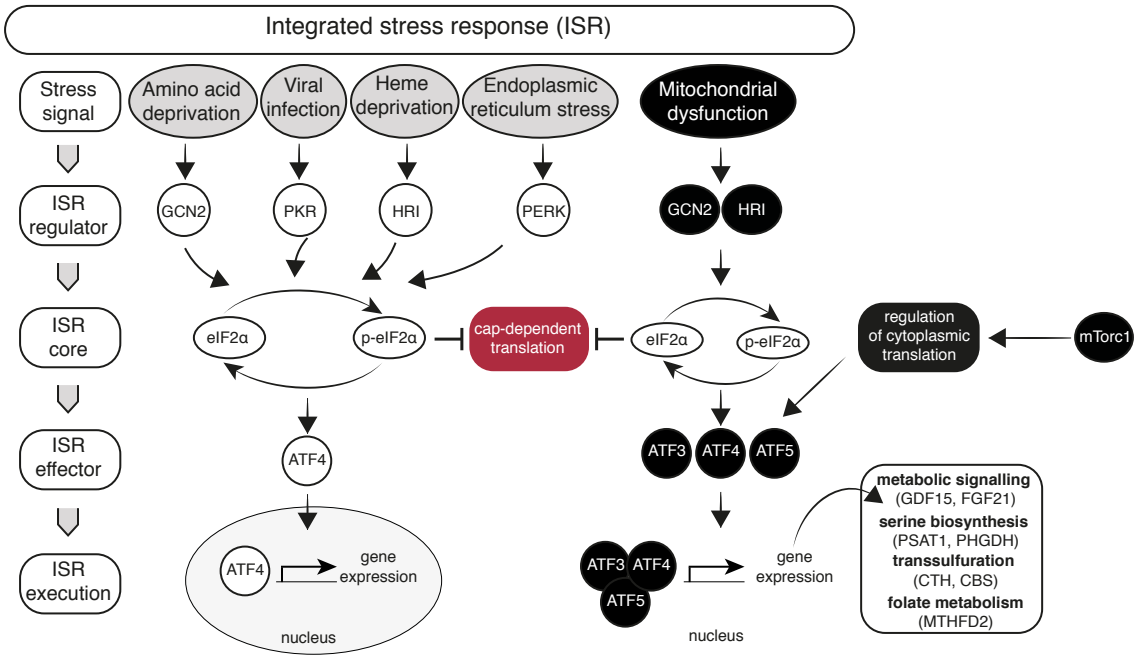

Figure 12. Schematic of the integrated stress response signalling (based on (Pakos-Zebrucka et al. 2016) and the primary research articles discussed in this chapter).

The key event in ISR signalling is the remodelling of cytoplasmic translation (Figure 12). Eukaryotic Initiation Factor 2 (eIF2) is the protein complex required for initiation of translation, and is dependent on the interaction with a modified guanosine at the 5' end of the mRNA (5' cap), or cap-dependent translation. Phosphorylation of the regulatory subunit **α** of the eIF2 complex (eIF2**α**) blocks cap-dependent translation and simultaneously activates cap-independent translation, including that of activating transcription factor 4 (ATF4) (Figure 12). ATF4 is implicated as the main mediator of the ISR execution, as it relocates to the nucleus and induces expression of its target genes (Figure 12). The ISR in eukaryotes is regulated by four kinases that display a rapid response to phosphorylate eIF2**α** upon stress (Taniuchi et al. 2016). Each kinase is associated with a distinct type of stress (Figure 12). In addition to these stereotypical functions, kinases also display a functional redundancy and synergistic activity, for example upon glucose deprivation (reviewed in (Pakos-Zebrucka et al. 2016)).

Mitochondrial dysfunction induces expression of genes associated with ISR (Silva et al. 2009; Moisoi et al. 2009; Tyynismaa et al. 2010; Richter et al. 2013; Dogan et al. 2014; Michel et al. 2015; Ost et al. 2015; Nikkanen et al. 2016; Bao et al. 2016; Kühl et al. 2017; Quirós et al. 2017; N. A. Khan et al. 2017; Forsström et al. 2019; Murru et al. 2019; Mick et al. 2020). This

was demonstrated in response to pharmacological OXPHOS inhibitors and genetic dysfunction in a variety of models including cultured cells and mice, as well as human patients. ISR effectors upon mitochondrial dysfunction appear to include ATF3, ATF4, and ATF5 (Figure 12). The regulation and the full range of cell responses to mitochondrial dysfunction in mammalian cells is only just beginning to be understood. It was suggested that responses to mitochondrial dysfunction are either regulated via non-ISR signalling mechanisms, or not controlled by the action of individual ISR-related kinases (Quirós et al. 2017; Ben-Sahra et al. 2016; Münch and Harper 2016). Other research however established the regulatory role of two eIF2**α** kinases (GCN2 and HRI) in ISR induction in cultured cells treated with OXPHOS inhibitors (Figure 12) (Michel et al. 2015; Fessler et al. 2020; Mick et al. 2020; Guo et al. 2020). The elucidation of stress response regulation *in vivo* is hindered by its rapid induction. Thus, studies using mouse models with chronic mitochondrial dysfunction have mostly focused on the resulting gene expression and metabolic signature.

The gene expression signature induced upon mitochondrial dysfunction is tissue-specific in mammals (Moisoi et al. 2009; Michel et al. 2015; Nikkanen et al. 2016; Forsström et al. 2019). This may be explained by both the differential regulation of the early stress response events (for example, actions of distinct kinases), and by the fact that targets of a transcription factor executing the response can be cell-specific. Chronic mitochondrial dysfunction can also lead to activation of many secondary responses. Mouse models with mild mitochondrial dysfunction, such as Deletor mice (see 2.3.6.1), allow the investigation over the time-course of cell responses. In a series of studies, it was established that the induced gene expression signature not only differs between the tissues, but also is temporarily regulated (Tyynismaa et al. 2010; Nikkanen et al. 2016; Forsström et al. 2019). Early stages included the induction of the folate cycle and the secreted growth/differentiation factor 15 (GDF15) and fibroblast growth factor 21 (FGF21), progressing to upregulation of serine biosynthesis and transsulfuration pathways (Forsström et al. 2019). The same group earlier found that FGF21 and GDF15 induction are specific to a molecular defect in mitochondrial gene expression but not OXPHOS complex subunit defects in human patients (Lehtonen et al. 2016). In mice, muscle-specific *Cox10* knockout however resulted in elevated FGF21 and MTHFD2 levels (Tyynismaa et al. 2010). Since the gene expression signature in response to chronic mitochondrial dysfunction was partially overlapping with ISR, it was named ISR^{mt} (integrated stress response mitochondrial). This terminology is used throughout this thesis. The core ISR^{mt} gene expression signature includes transcription factors ATF3-5, as well as factors that mediate metabolic signalling (GDF15, FGF21), serine biosynthesis (PSAT1, PHGDH), transsulfuration (CTH, CBS), and folate metabolism (MTHFD2). The promoters of several ISR^{mt} genes harbor a sequence known as amino acid starvation response element (Tyynismaa et al. 2010), as it was first described in regulation of the transcriptional response to starvation (Figure 12, amino acid deprivation). The response is also associated with changes in metabolite levels, such as increased serine and glycine levels, as well as purine biosynthesis and degradation molecules (Nikkanen et al. 2016; Bao et al. 2016).

Another contributor to remodelling of cytoplasmic translation upon mitochondrial dysfunction is the regulation exerted by the key nutrient sensor of the cell, a protein complex named mammalian target of rapamycin complex 1 (mTorc1) (Johnson et al. 2013; Ben-Sahra et al. 2016; N. A. Khan et al. 2017; Quirós et al. 2017; Barriocanal-Casado et al. 2019). Targets of mTorc1 include a subunit of eIF2 complex (4E-BP1) and a ribosomal protein p70-s6 kinase 1 (S6K). 4E-BP1 acts as a translation inhibitor, and its phosphorylation by mTorc1 releases 4E-

BP1 from the eIF2 complex, thus promoting translation. S6K is activated by phosphorylation, and can stimulate the initiation of translation through phosphorylation of the S6 ribosomal protein. *Ndufs4* knockout or expression of a pathogenic *Twnk* variant in mice resulted in upregulated S6 phosphorylation, which was regulated by mTorc1 (Johnson et al. 2013; N. A. Khan et al. 2017). ISR regulators were also proposed to modulate mTorc1 activity, indicating a possible crosstalk of these pathways upon mitochondrial dysfunction (Condon et al. 2021).

Collectively, this body of research established that cells respond to mitochondrial dysfunction with changes in gene expression and metabolism. These responses can clearly be important for pathogenesis, but their regulation and significance have only begun to be elucidated.

2.3.6.4 Strategies to combat mitochondrial diseases

Therapeutic approaches to mitochondrial diseases aim to either correct the molecular defect (enzyme or mitochondrial replacement therapy), to alleviate specific symptoms (for example, anticonvulsants in the treatment of epileptic seizures), or to govern adaptation (for example, dietary interventions or targeting cell stress responses) (reviewed in (Suomalainen 2011; Russell et al. 2020; C. Viscomi and Zeviani 2020)). The challenges inherent to treating mitochondrial disease lie in their diverse clinical and genetic background, severity, and involvement of multiple organ systems.

Preclinical studies are limited by the few existing animal models of mitochondrial diseases, and treatment approaches will likely tend to be extrapolated from rather distant models and diseases. Several treatments extended the lifespan of *Ndufs4* knockout mice (Leigh disease model, 2.3.6.2): i) administration of rapamycin, a drug which inhibits mTorc1 activity (Johnson et al. 2013); ii) chronic exposure to hypoxia (Jain et al. 2016); iii) administration of nicotinamide mononucleotide (NAD^+ precursor) or dimethyl **α**-ketoglutarate (C. F. Lee et al. 2019); iv) inhibition of mitochondrial genome expression with antibiotics (Perry et al. 2021). Inhibition of mitochondrial genome expression was earlier shown to modulate phenotypes upon mitochondrial translation stress and deficiency of mitochondrial protease AFG3L2 function (Richter et al. 2015, 2013, 2019). Notably, none of these treatments restored the OXPHOS function impaired by NDUFS4 deficiency, highlighting the importance of targeting mechanisms of adaptation to mitochondrial dysfunction. NAD booster therapy was earlier applied also in Deletor mice (N. A. Khan et al. 2014). Below I discuss findings on the administration of rapamycin and a ketogenic diet, that are most relevant for the research presented in this thesis.

Rapamycin. Rapamycin is a compound produced by the bacteria *Streptomyces hygroscopicus* that inhibits mTorc1 activity. Rapamycin administration has pleiotropic consequences, including immunosuppressive action, antiproliferative effects, and autophagy stimulation (reviewed in (Y. C. Kim and Guan 2015; G. Y. Liu and Sabatini 2020)). Rapamycin extends lifespan in yeast, worms, flies, and mice (reviewed in (Arriola Apelo and Lamming 2016; G. Y. Liu and Sabatini 2020)). It is an approved drug used for treatment of the lung disease lymphangioleiomyomatosis (McCormack et al. 2011; Bissler et al. 2008) and in organ transplantation. Adverse effects of chronic rapamycin administration include decreased sensitivity to insulin and glucose tolerance (Lamming et al. 2012; Teutonico, Schena, and Di Paolo 2005; Johnston et al. 2008; Houde et al. 2010). The second generation of mTorc1 inhibitors can however reduce these side effects (reviewed in (Dumas and Lamming 2020)).

Daily injection or dietary administration of rapamycin extended the lifespan of *Ndufs4* knockout mice, although did not rescue the growth defect (Johnson et al. 2015; Felici et al. 2017; Johnson et al. 2013). Rapamycin administration resulted in decreased weight gain also in wild type mice (Johnson et al. 2015; Siegmund et al. 2017). The treatment decreased the occurrence of neurological symptoms, prevented formation of brain lesions and attenuated reactive gliosis (assessed by GFAP and IBA1 protein levels) (Johnson et al. 2013). Rapamycin administration was also effective in other animal models with mitochondrial dysfunction (the models are introduced in 2.3.6.2): i) in Deletor mice, it inhibited ISR^{mt} and reduced the number of muscle fibers with OXPHOS deficiency (N. A. Khan et al. 2017); ii) in mice with thymidine kinase 2 dysfunction, it extended lifespan, but did not affect the morbidity (Siegmund et al. 2017); iii) in mice with muscle-specific *Cox15* knockout, it improved the motor phenotype (Civiletto et al. 2018). Rapamycin administration to mice with *Coq9* pathogenic variant however exacerbated the phenotype, resulting in accelerated body weight loss, decreased survival and increased brain lesions including reactive gliosis (Barriocanal-Casado et al. 2019).

The described above effects of rapamycin administration to mouse models with mitochondrial dysfunction appeared to be independent of the level of mTorc1 activity in untreated mice. In mice with disrupted function of *Ndufs4*, *Polg,* and thymidine kinase 2 the mTorc1 activity was increased and rapamycin administration had beneficial effects (N. A. Khan et al. 2017; Johnson et al. 2013; Siegmund et al. 2017). *Coq9* knock-in mice also displayed an increased mTorc1 activity, however rapamycin administration had detrimental effects (Barriocanal-Casado et al. 2019). Mice with muscle-specific *Cox15* deficiency did not display an increased mTorc1 activity, however rapamycin administration modulated the morbidity of this model (Civiletto et al. 2018). Of note, as the main readout of mTorc1 activity, in most of the aforementioned studies was a phosphorylation level of ribosomal protein S6, which is not a direct target of mTorc1 and is phosphorylated by other kinases (see 2.3.6.3). Taken together, these studies showed that rapamycin can have beneficial or detrimental effects to modulate morbidity phenotypes caused by mitochondrial dysfunction.

Ketogenic diet. Ketogenic diets have a high-fat, moderate protein and low-carbohydrate composition. This limits glucose availability and stimulates compensatory gluconeogenesis and fatty acid oxidation in the liver, leading to production of ketone bodies (**β**-hydroxybutyrate, acetoacetate, and acetone). Ketone bodies are taken up by other organs including the brain, and metabolized by both mitochondrial and cytoplasmic enzymes to serve as an alternative energy source when the availability of glucose is limited. Ketogenic diets display anti-epileptic properties and are prescribed on an elective basis to patients who manifest with drug-resistant epilepsy, including patients with mitochondrial diseases. The clinical experience of administering ketogenic diets to patients with mitochondrial diseases is reviewed below.

For 10 out of 14 children diagnosed with deficiencies in OXPHOS complexes, ketogenic diets resulted in a decreased frequency or the elimination of seizures (H.-C. Kang et al. 2007). Several patients however had adverse effects and were advised to discontinue the diet, and 2 out of 14 patients died of pneumonia-related complications during the diet administration (H.-C. Kang et al. 2007). In another study, ketogenic diets were administered to 24 patients with intractable epilepsy and OXPHOS dysfunction (Y. M. Lee et al. 2008). Three patients discontinued the diet due to serious infections, however 18 patients had seizure reduction of more than 50%, and 12 patients became seizure free (Y. M. Lee et al. 2008). The limitation in interpreting these studies is that patients had diverse genetic diagnoses.

Ketogenic diets were administered also to seven children with dysfunction of the pyruvate dehydrogenase complex that catalyses the conversion of pyruvate to acetyl-CoA (Wexler et al. 1997) (the role of acetyl-CoA is discussed in 2.1.4.1). All patients succumbed to the disease, but in two sets of siblings, a child who received a ketogenic diet earlier or whose diet had lower carbohydrate content, lived longer (Wexler et al. 1997). However, the effect on the frequency of seizures was not reported (Wexler et al. 1997). Another study found the ketogenic diet to decrease epileptic episodes or paroxysmal dystonia in patients with pyruvate dehydrogenase complex deficiency (Barnerias et al. 2010). Finally, ketogenic diets were reported to be tolerated and to temporarily improve manifestations for patients with pathogenic variants in *Polg,* including those diagnosed with Alpers-Huttenlocher syndrome (Cardenas and Amato 2010; A. Khan et al. 2012; Martikainen et al. 2012; Joshi et al. 2009).

The effects of ketogenic diets using animal models have been assessed for mitochondrial pathogenesis which manifests in muscle and liver (Ahola-Erkkilä et al. 2010; Purhonen et al. 2017). Deletor mice (see 2.3.6.2) tolerated the ketogenic diet for 40 weeks without evident adverse effects (Ahola-Erkkilä et al. 2010). The diet resulted in a reduced number of muscle fibers with OXPHOS deficiency, and elimination of the ragged red fibers with accumulations of abnormal mitochondria (Ahola-Erkkilä et al. 2010). Administration of ketogenic diet to patients with mitochondrial myopathy resulted in selective lysis of ragged red fibers (Ahola et al. 2016). Patients however had progressive symptoms including burning sensations and muscle pain, and the administration of the ketogenic diet was discontinued 4-11 days after the start of the trial (Ahola et al. 2016). The elimination of ragged red fibers in Deletor mice and in human patients could have occurred via a similar mechanism, however, in mice the administration of a ketogenic diet for seven days did not result in muscle degeneration (Ahola et al. 2016). In mice with disrupted Complex III assembly, the ketogenic diet resulted in delayed liver fibrosis and reduced inflammation (Purhonen et al. 2017), signifying the beneficial effects.

The molecular basis of anti-seizure effects of the ketogenic diets remains mostly elusive and is a subject of debate, but was recently linked to indirect modification of the brain neurotransmitter levels via microbiome metabolism (Olson et al. 2018). The robust effects of ketogenic diets on metabolism call for the special attention in its administration to patients with metabolic syndromes, including patients diagnosed with mitochondrial diseases.

2.4 Gene inactivation in the central nervous system using Cre-Lox recombination

Manipulating the expression level of individual genes provides an approach to study functional consequences of protein activity. Abolishing gene expression can be useful to understand the functions (or, strictly speaking, consequences of dysfunction) of an encoded protein. This also gives an insight to the pathogenesis caused by compromised protein functions. Constitutive and ubiquitous inactivation of genes in model organisms often leads to lethality during development, which impedes research inquiries (see 2.3.6.2). To disrupt expression of a gene in a specific cell type *in vivo*, the Cre-Lox recombination system is widely used. The system was initially discovered in the bacteriophage P1 (Sternberg and Hamilton 1981) and consists of an enzyme Cre recombinase, which induces a DNA recombination event between a pair of short sequences, known as loxP sequences. To induce a gene knockout, a construct with loxP sites placed around key exons or regions that control gene expression is targeted to the genome. Expression of Cre recombinase in cells harboring loxP sites leads to excision of the DNA fragment between the sites, preventing expression of a functional protein. Once the system was proven to be effective in mammalian cells (Sauer and Henderson 1988) and in mice (H. Gu et al. 1994), it provided a revolutionary toolbox for biological research. Drivers to direct Cre expression under cell-specific promoters quickly became used in neuroscience to manipulate gene expression in the cell types of interest (Gong et al. 2007). There are several methodological considerations for using Cre-Lox recombination in the CNS:

a) ***Autonomous effects of transgene integration and expression.*** Expression of Cre recombinase can have consequences for cell homeostasis, and the transgene integration can affect the expression of endogenous genome components (reviewed in (Harno, Cottrell, and White 2013; McLellan, Rosenthal, and Pinto 2017; Wüst, Houtkooper, and Auwerx 2020)). To control for unintended effects, mice that harbor a Cre expression cassette but no loxP sites can be considered as controls. This however still does not rule out a situation when transgene-induced effects are normally undetectable, but exacerbated by a gene knockout. A commonly used approach to generate transgenic mouse strains is a pronucleus injection leading to integration of the construct or its multiple copies into a random locus in the genome. Good practices involve characterisation of the phenotype of generated strains and defining the site of integration. Site-directed integration of the construct into a defined gene locus, such as ROSA26 locus (Friedrich and Soriano 1991), also may be preferred. Examples of unintended phenotypes of Cre-expressing drivers include a nestin-Cre strain, which manifests with a metabolic and behavioral phenotype (Declercq et al. 2015) and mice with Cre driven by a cardiac **α**-myosin heavy chain promoter, which manifest with cardiomyopathy (Buerger et al. 2006; Pugach et al. 2015).

b) ***Spontaneous and induced Cre expression.*** Spontaneous expression of Cre recombinase (directed by a promoter of choice) typically occurs during embryogenesis or postnatal development. Once the recombination event occurs, the DNA of a cell and its progeny will remain recombined. To achieve temporal regulation of the recombinase activity, systems such as the Cre estrogen receptor (CreER) variant and the tetracycline-controlled transcriptional activation are used. CreER harbors a mutated ligand binding domain of the estrogen receptor and is shuttled to the cytoplasm. Administration of an estrogen receptor modulator tamoxifen, a product of which binds to the estrogen receptor, stimulates the translocation of Cre to the nucleus to induce a recombination event. In the tetracycline-controlled system, Cre expression is controlled by a promoter with a tetracycline response

element, and the construct encodes for a tetracycline repressor protein from *Escherichia coli* fused with an activation domain from a herpes simplex virus protein. The resulting protein can bind to the tetracycline response element, and this binding is induced or inhibited by the presence of tetracycline and its derivatives (Tet-Off and Tet-On systems). In the described above systems recombinase activity only occurs upon tamoxifen or tetracycline administration, providing a tool for the temporal control of gene expression. Considerations include leakiness of the systems (reviewed in (Harno, Cottrell, and White 2013; McLellan, Rosenthal, and Pinto 2017; Wüst, Houtkooper, and Auwerx 2020)) and autonomous effects of these drugs: tamoxifen affects physiology through its modulation of the estrogen receptor (reviewed in (Harno, Cottrell, and White 2013; McLellan, Rosenthal, and Pinto 2017; Wüst, Houtkooper, and Auwerx 2020)); and tetracyclines act to inhibit mitochondrial genome expression (Moullan et al. 2015). The efficacy of recombination events is also dependent on the protocols to administer Cre-activating compounds (Jahn et al. 2018).

c) ***Expression pattern within the CNS.*** Each mouse strain generated by a random insertion of a transgene is unique, and patterns of expression even when using identical promoter elements can vary. Variations of a promoter region and usage of promoters derived from different species generate additional variability. A prominent example of this is a variety of GFAP-Cre strains (Zhuo et al. 2001; Gregorian et al. 2009; A. D. R. Garcia et al. 2004; Ganat et al. 2006; Hirrlinger et al. 2006). Each strain has a unique spatiotemporal pattern of expression, targeting Cre to subpopulations in different regions. Transgene expression can also be affected by the genetic background of the mouse strain (Bai et al. 2013).

d) ***Expression pattern outside of the CNS.*** Genes, the expression of which is restricted to a cell type of interest within the CNS, may be expressed in other tissues (reviewed in (McLellan, Rosenthal, and Pinto 2017)). For example, genes that are used to target astrocytes (GFAP, ALDH1L1, aquaporin 4, and EEAT1) are also expressed in peripheral tissues (data from JAX database). Additionally, many Cre-expressing mouse lines intended to target Cre expression into the CNS are expressed in the germline, which may result in heterozygous progeny in crosses (Luo et al. 2020). To decrease unintended expression in peripheral tissues, viral delivery of Cre recombinase by intracranial injections can be used. For strains with germline expression of Cre, Cre-positive breeders only of the sex that does not possess such expression can be used, or an appropriate genotyping of progeny can be carried out.

In summary, Cre-Lox recombination is a valuable tool to manipulate gene expression, which has been widely used in biological research during the last decades. It is important to consider the limitations of this system, and, where possible, act to circumvent such limitations by using appropriate controls, validating phenotypes with more than one Cre driver, as well as to apply complementary approaches to manipulate gene expression.

3 AIMS OF THE STUDY

Statement of the research question: the general aim of this thesis was to investigate the pathogenesis arising from mitochondrial dysfunction in the central nervous system, with a particular emphasis on the role of astrocytes. The specific aims were:

i) To investigate the primary consequences of mitochondrial dysfunction in neurons and astrocytes.

ii) To test the efficacy of treatment approaches to modulate the pathology caused by astrocytic mitochondrial dysfunction.

iii) To elucidate astrocytic responses to mitochondrial dysfunction.

4 MATERIALS AND METHODS

Methods used in the research presented in this thesis, with which I was personally involved, are summarised in Table 1. Detailed materials and methods are presented in the manuscripts (I-III) or in this chapter, and several methodological considerations are discussed below. Experimental procedures that required the use of laboratory animals were approved by The National Animal Experiment Review Board and Regional State Administrative Agency for Southern Finland, following the European Union Directive.

Table 1. The list of main methods used in this thesis.

METHOD	STUDY
Genetically modified mouse models	(I-III), unpublished data
Hematoxylin and eosin stain	(I-II), unpublished data
Immunohistochemistry	(I-III), unpublished data
Fluorescence-RNA *in situ* hybridization	(III)
Transmission and scanning electron microscopy	(I, III)
Astrocyte purification from the adult mouse brain	(III)
Astrocyte cultures	unpublished data
DNA extraction and mtDNA quantification	(I-III)
RNA extraction and RT-qPCR	(I-III)
Analysis of RNA sequencing data	(III), unpublished data
Protein extraction and immunoblot	(I, II)
Metabolite extraction and analysis of metabolomics data	(II)
Molecular cloning	(III)
Viral transduction, cultured cells and *in vivo*	(III), unpublished data
Lipid stain	unpublished data
Statistical analyses	(I-III)

4.1 Mouse models used in the study

In the research presented in this thesis, Cre-Lox recombination system was used to induce conditional knockout of mitochondrial genes in mouse brain neurons and astrocytes:

1. TwKO^{astro} and TwKO^{neuro} are mice with perturbed mitochondrial gene expression, caused by the cell-specific knockout of *Twnk*, encoding replicative mtDNA helicase Twinkle, in astrocytes (TwKO^{astro}) or in neurons (TwKO^{neuro}). These mice were generated before the research towards this thesis was initiated, characterised in (I), and used in (I-III).

Generation of mice harboring loxP sequences in the endogenous locus of *Twnk* is described elsewhere (Nikkanen et al. 2016). LoxP sequences are located in the introns 2 and 4 of *Twnk*, and Cre-mediated recombination between these sites produces a frame-shift event, leading to a stop codon. These mice also harbor Y508C mutation in the exon 3 of *Twnk*, which is analogous to a human pathogenic variant. Twnk$^{Y508C/Y508}$ mice were asymptomatic and indistinguishable from wild type mice at the age used (up to 9.5 months of age). For the research presented in the study (III), we also generated mice harboring loxP sites in *Twnk* gene, but without any pathogenic variant (Twnk$^{loxp/loxp}$), using the same approach as previously described (Nikkanen et al. 2016). Phenotypes of TwKO^{astro} mice on Twnk$^{lY508C/Y508}$ and Twnk$^{loxp/loxp}$ genetic background were indistinguishable. To induce astrocytic knockout of *Twnk* (TwKO^{astro}), mice harboring loxP sequences in *Twnk* were crossed to Gfap73.12-Cre mice (JAX: 012886). To induce neuronal knockout of *Twnk* (TwKO^{astro}), mice harboring loxP sequences in *Twnk* were crossed to CamKII**α**-Cre mice (JAX: 005359).

2. Cox10KO^{astro} are mice with OXPHOS deficiency in astrocytes, caused by the cell-specific knockout of *Cox10* gene encoding COX10 protein. COX10 is heme A: farnesyltransferase, involved into heme A maturation, the function of which is essential for Complex IV assembly and function. Presented findings using this model are unpublished.

Mice harboring loxP sites in *Cox10* gene were generated elsewhere (Diaz et al. 2005), and for this study were kindly provided by Prof. Howard Jacobs, Tampere University, Finland. In these mice, the exon 4 of *Cox10*, which harbors the active site of COX10 protein, is flanked by loxP sequences (Diaz et al. 2005). To induce astrocytic knockout of *Cox10* (Cox10KO^{astro}), mice harboring loxP sequences in *Cox10* gene were crossed to Gfap73.12-Cre mice (JAX: 012886).

Cre expression. The induction of Cre recombinase in the strain described above is spontaneous: Gfap73.12-Cre expression starts at the postnatal day 4 (A. D. R. Garcia et al. 2004); CamKII**α**-Cre expression starts during the third to fourth postnatal week (Tsien et al. 1996)). Expression of Gfap73.12-Cre is confirmed in cortical astrocytes, hippocampal astrocytes, cerebellar astrocytes and Bergmann glia, ependymal cells, astrocytes of the subventricular zone, adult neural stem cells, olfactory bulb, and in the spinal cord ((A. D. R. Garcia et al. 2004), JAX repository, (I, III)). The transgene is also expressed in the progeny of neural stem cells, and in a number of cortical, hippocampal, cerebellar, and midbrain neurons (JAX repository and unpublished own observations). For TwKO^{astro} and Cox10KO^{astro} mice, the research presented in this thesis was focused on astrocytes. Expression of CamKII**α**-Cre is confirmed in a subpopulation of excitatory forebrain neurons and in the pyramidal layer of the hippocampus (Tsien et al. 1996). Both Gfap73.12-Cre and CamKII**α**-Cre may also be expressed

in peripheral tissues (JAX repository, and for Gfap73.12-Cre also unpublished own observations). The research presented in this thesis was focused on phenotypes within the CNS.

Due to the expression of Cre in the male germline (JAX repository), both *Twnk*$^{loxp/loxp}$ and Twnk$^{loxp/null}$ were generated in these crosses and used interchangeably, as mice were indistinguishable. Littermates were used as control mice, and are referred to throughout this thesis as Ctrl mice. Both female and male mice were used in the research presented in this thesis (for details, refer to I-III). Gfap73.12-Cre reporter mice were generated by crossing Gfap73.12-Cre to Ai14 tdTomato mice, where expression of tdTomato is controlled by a floxed stop cassette (JAX: 007914). Mice on C57Bl/6OlaHsd or mixed genetic background mice were used (mixed background originated from reporter mice and newly derived Twnk$^{loxp/loxp}$ mice).

4.2 Astrocyte purification from the mouse brain

Methodological considerations. Protein and mRNA levels in tissue lysates are contributed by different cell types. Approaches to purify cell populations for downstream gene expression analyses include microdissection from tissue slices and sorting from a tissue-derived cell suspension. Microdissection allows the isolation of cells from intact tissue, but is laborious and typically yields a low amount of material. Cell suspension-based techniques are quicker and yield more material, but include mechanical or enzymatic disruption of the tissue, which can induce changes in gene expression (Hammond et al. 2019; Mattei et al. 2020). Single-cell analysis is also a powerful suspension-based technique, but the downstream analyses typically result in the incomplete genome coverage (reviewed in (Armand et al. 2021)). Single-cell approaches also may result in disproportionate capturing of cell populations. For example, many published brain single-cell analyses have an underrepresented astrocyte population (reviewed in (Liddelow, Marsh, and Stevens 2020)). All these approaches typically result in enrichment of mostly cell soma content. To analyse other cell parts or specific organelles, corresponding molecular tags can be used.

A challenge to purify cells from the adult brain is the cell density and complex three-dimensional interactions between the cell types. Disruption of intercellular connections yields high amounts of cell and myelin debris, which may impede further purification. To circumvent this, filtering steps, differential centrifugation, negative immunoselection of unwanted cell populations, as well as antibody-based removal of myelin debris are used (J. A. Garcia, Cardona, and Cardona 2014; Szuchet, Arnason, and Polak 1980). To purify astrocytes, fluorescence-activated sorting, immunopanning, and magnetic-activated sorting are used (Cahoy et al. 2008; Foo 2013; Batiuk et al. 2017). All these approaches will yield a subpopulation of astrocytes depending on the gene promoter or an antigen used. Antibody-based approaches may also be impeded if the antigen expression changes in the experimental condition.

Implementation. To analyse transcriptional responses of astrocytes to mitochondrial dysfunction (III), astrocytes were purified from 3-3.5 months old TwKO^{astro} and Ctrl brain cortexes using magnetic-activated sorting with magnetic beads coated with the ACSA-2 antibody, which recognises the astrocyte-specific surface antigen ATP1B1 (Leanne Melissa Holt and Olsen 2016; Batiuk et al. 2017). Astrocytes were successfully enriched in the ACSA-2$^+$ fraction from both Ctrl and TwKO^{astro} mice, as assessed by qPCR analysis of cell-type specific markers (Figure 13).

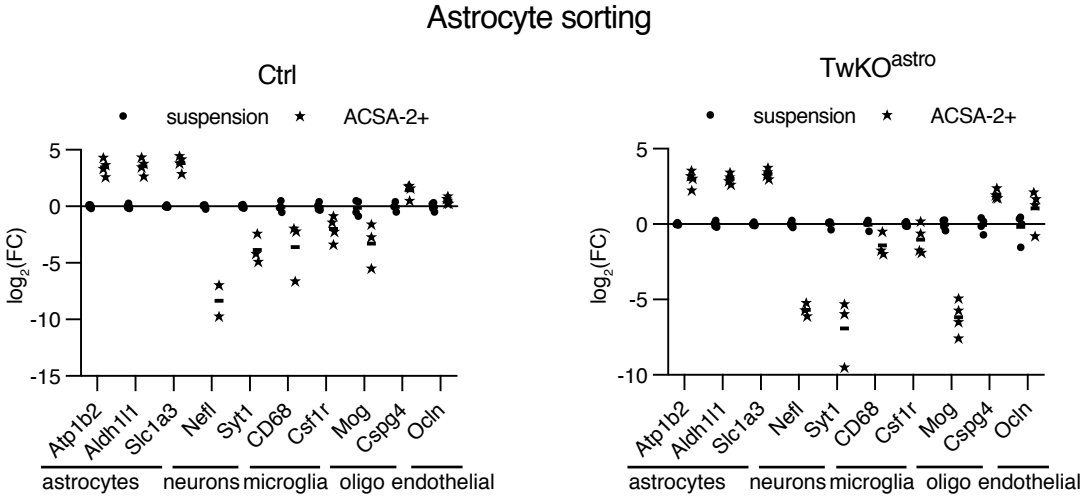

Figure 13. The efficacy of astrocyte purification from the adult mouse brain (modified from (III)). Expression of cell-specific markers, measured using RT-qPCR. ACSA-2$^+$ is the final fraction enriched for astrocytes, suspension is the cell suspension after mechanical and enzymatic digestion of the brain. Symbols represent individual samples. FC = fold change, relative to suspension. Oligo = oligodendrocyte lineage cells. ACSA-2$^+$ = astrocyte cell surface antigen-2.

4.3 Astrocyte cultures

Methodological considerations. Cultured cells provide the system to investigate cell biology of the CNS with high resolution. The cell culture models include primary cells, cell lines, and cells differentiated from induced pluripotent stem cells. To investigate pathogenesis of mitochondrial dysfunction, a relevant approach would be to culture cells derived from the mouse models upon manifestation. However, the viability of cells derived from the brain drastically decreases with age of the mouse. This is likely due to the vulnerability of the cells to disruption of intercellular connections, removal from a cell niche, and possibly due to the presence of debris and toxic factors released by dying cells into suspension. Consequently, astrocyte cultures are typically derived from neonatal pups.

The original method to derive nearly pure astrocyte cultures was developed in 1980 (McCarthy and de Vellis 1980), and with minor modifications is still extensively used. The method is based on culturing cells derived from the cortex of neonatal pups by enzymatic digestion (McCarthy and de Vellis 1980). These cultures do not have many viable neurons, but consist of astrocytes, oligodendrocytes, and can contain microglia. Astrocytes proliferate and after several days form a monolayer, after which non-astrocytic cells can be removed by agitating cell culture flasks on a shaker for several hours (McCarthy and de Vellis 1980). While

the technique was invaluable for pioneering astrocyte research, it was evident that critical properties of cultured astrocytes differed dramatically from those *in vivo*. Cultured astrocytes were actively dividing, did not extend cell processes, and acquired amoeboid, flat, fibroblast-like morphology. This is attributed mainly to the presence of serum in the cell culture medium. Serum helps to maintain cell viability, but stimulates astrocytes to re-enter the cell cycle.

It was not until 2011 that an approach to derive pure astrocytes cultures that better resemble *in vivo* astrocyte properties was developed (Foo et al. 2011). This technique used antibody-based affinity for serial depletion of non-astrocytic cell populations and for a final enrichment of the astrocyte population. Identification of factors that promote astrocyte survival allowed to maintain viability of cultured astrocytes without adding serum into the medium (Foo et al. 2011). Of critical importance was an addition of the heparin-binding epidermal growth factor (HBEGF), a ligand for growth factors receptors including epidermal growth factor receptor (EGFR). Astrocytes cultured in this chemically defined medium without added serum divided only several times and extended multiple thin processes, better resembling the appearance of astrocytes *in vivo* (Foo et al. 2011). Compared to astrocytes cultured in serum-containing media, these cultures better promoted synapse formation when co-cultured with neurons (Foo et al. 2011). Subsequent transcriptome analysis revealed that astrocytes cultured in the presence of serum acquire gene expression signature corresponding to reactive astrogliosis, while transcriptome of astrocytes cultured in the chemically defined medium is more similar to astrocytes purified from the mouse brain (Y. Zhang et al. 2016). It was also established that after initial plating in the full medium, removal of HBEGF or inhibition of EGFR signalling promotes the maturation of cultured astrocytes (Jiwen Li et al. 2019).

Implementation. In the research presented in this thesis, I used primary astrocyte cultures derived from neonatal pups to induce *Twnk* knockout. I modified the technique from (Foo et al. 2011) to purify astrocytes from neonatal mouse cortices (postnatal day 3-5) using antibody-coated magnetic beads instead of immunopanning (similarly to 4.2), and cultured astrocytes in the chemically defined media as in (Foo et al. 2011). While this research was being conducted, a similar approach was published elsewhere (Leanne M. Holt et al. 2019). To investigate how the presence of serum affects astrocyte properties, I purified astrocytes using ACSA-2 magnetic beads and plated them in chemically defined medium with no added serum. After one day in culture, I either replaced the 50% of the medium either fresh chemically defined medium without serum, or with chemically defined medium containing 10% fetal bovine serum to analyse cultures two days after (Figure 14). As expected, astrocytes cultured in the presence of serum were proliferating and had fibroblast-like morphology (Figure 14). Cultured in these conditions astrocytes extended multiple long processes and were slow cycling, similarly to immunopanned astrocytes cultured in the medium with analogous composition (Figure 14, (Foo et al. 2011)). Astrocytes cultured in the serum-free medium had lower GFAP expression compared to cultures with added serum (Figure 14).

Additionally, I found that the mitochondrial network differed profoundly between culture conditions (Figure 14). In the presence of serum, the mitochondrial network was reticular (Figure 14). In astrocytes cultured in the chemically defined media devoid of serum, the network was fragmented, mitochondria appeared as short tubules and were bulbous (Figure 14). This exemplifies the dynamic nature of a mitochondrial network (see 2.3). It would be of interest to investigate which factors control such remodelling in these conditions, how mitochondrial function is affected, and whether the mitochondrial network in serum-containing

media corresponds to changes occurring in pathologies associated with disruption of the blood-brain barrier (for example, trauma and stroke). Interestingly, remodelling of the mitochondrial network was reported to occur upon stab wound injury *in vivo* (Göbel et al. 2020).

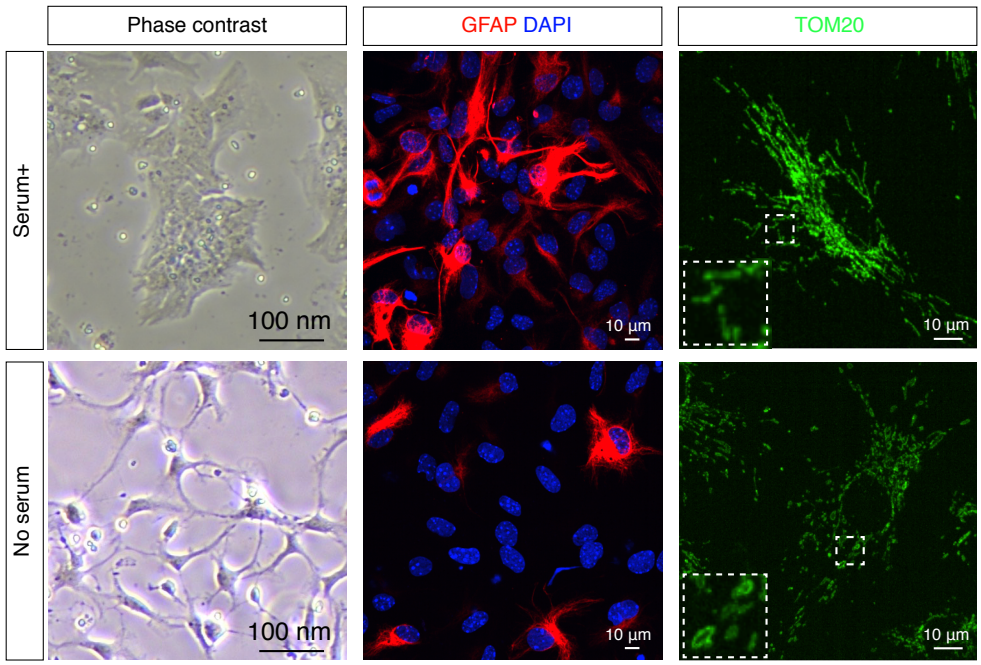

Figure 14. Astrocytes cultured in the medium with or without added serum. Astrocytes were purified from neonatal mouse cortices using magnetic beads coated with ACSA-2 antibody (similarly to 4.3). After one day in culture, the medium with 10% fetal bovine serum was added to Serum+ cultures. Cells were cultured for two more days, imaged with phase contrast microscopy, and fixed for immunostainings against GFAP and TOM20. Square indicates the area shown as an inset in the bottom left corner. Immunofluorescence: MIP, confocal images.

In summary, the modification of a published protocol to purify astrocytes from the neonatal mouse brain yielded viable astrocytes cultures. Astrocyte cultures in the chemically defined medium with no added serum were more similar to astrocytes *in vivo*, and thus were used for the research presented in this thesis.

Detailed protocol. Pups were decapitated, brain cortexes were dissected and dissociated using an enzymatic kit (Neural Tissue Dissociation Kit (P), Miltenyi Biotec #130-092-628). Astrocyte fraction was enriched using Anti-ACSA-2 MicroBead Kit (Miltenyi Biotec #130-097-679). Glass coverslips (Engelbrecht GmbH #K12222 or #K11818) were coated with Matrigel (Corning #354277) or laminin (Sigma-Aldrich #CC095). 20-50x10^3 cells were pre-plated in 50 µl volume of cell culture media, and incubated for 15 minutes in a cell culture incubator. After this, 500-1000 µl of cell culture media was added to 6-well cell culture plates. Approximately 50% of the cell culture media was changed every three to four days. Viral transduction was performed on a second to fourth day in culture. Adenoviral vectors were purchased from the University of Iowa Viral Vector Core (Ad5Cmv-ntGFP and Ad5CMVCre-eGFP). For transduction, the virus was diluted in 50% fresh 50% conditioned cell culture media in half of the normal culturing volume, and cells were incubated with the virus for four hours in the cell culture incubator (multiplicity of infection was 20-50). After this, the medium was removed,

cells were rinsed with the basic medium, and 50% fresh 50% conditioned cell media was added. Individual litters were used to generate independent experiments. For RNA extraction, the medium was removed, cells were rinsed with PBS and immediately lysed on a plate using a lysis buffer (NucleoSpin RNA plus Macherey-Nagel #740984.250). RNA was extracted using RNA-binding columns according to manufacturer instruction (NucleoSpin RNA plus Macherey-Nagel #740984.250). For immunofluorescence, the medium was removed, cells were rinsed with PBS, fixed with 4% formaldehyde solution for 15 minutes at room temperature, rinsed with PBS, and stored under PBS.

4.4 Lipid staining

To stain lipids on mouse brain sections, a BODIPY compound was used (Thermo Fisher Scientific #D3922). This chemical has an intrinsic affinity to neutral lipids. To prepare a stock solution, BODIPY was dissolved in DMSO to a concentration of 1 ng/µl, aliquoted, and stored at -20 °C. Mice were terminally anesthetized with mebunat and transcardially perfused with ice-cold PBS, followed by perfusion with ice-cold 4% formaldehyde solution. Brains were postfixed overnight, incubated for several days in 30% sucrose and frozen in the embedding media and sectioned. Sections were incubated for 10 minutes in PBS, 10-60 minutes in BODIPY solution (1:100-1:1000 of the stock solution in PBS), washed twice for five minutes in PBS, mounted, and imaged the same day.

4.5 Statistical analyses

In the research presented in this thesis, the majority of experiments were designed to collect data on the distribution of a parameter of interest from two populations (for example, expression level of *Gfap* in Ctrl compared to TwKO^{astro} mice). Such data are presented as scatter plots, where all data points are plotted. To enhance visualisation, these plots are nearly always overlaid with box and whiskers plots (for example, Figure 16A). To make the interpretation of the effect size straightforward, nearly all data are presented as a fold change compared to a relevant control.

A modern tendency in biological research is to analyse any data of the type described above using statistical methods. Most often a Student's *t*-test to perform a null hypothesis statistical significance testing is used, from which a *p*-value is calculated. This *p*-value is then used to conclude whether the difference between populations is statistically significant. It has been extensively argued that if all data points are reported, using statistical testing is not necessary; and if the small samples sizes are used, the usage of *p*-value is skewed (Leek and Peng 2015; Ho et al. 2019; Singh Chawla 2017; Nuzzo 2014; Makin and de Xivry 2019). Consequently, it is discussed that computation of *p*-value may be replaced with estimation plots, complemented by calculation of the effect size, or omitted altogether. Parametric statistical tests typically rely on assumptions of normal distribution of data and homogeneity of variance between the groups. Testing for these assumptions does not appear to be meaningful with the sample sizes used here and commonly in similar research (for example, 4-10 mice per group). Nonparametric statistics, such as Mann–Whitney test comparing ranks, does not rely on such assumptions, but also has limited relevance for small sample sizes. Consequently, I conclude that for this type of data, plotting data as scatter plots is an appropriate way to allow the reader to evaluate data independently and perform pairwise comparisons (Weissgerber et al. 2015). Results of statistical analyses for such data are not reported, but can be found in (I-III), unless unpublished data are presented.

Data obtained with RNA sequencing and metabolomics analysis, both of which had a large number of pairwise comparisons, were analysed using established paradigms for this type of data. For differential expression analysis of RNA sequencing, I used Sleuth or Deseq2 packages(Pimentel et al. 2017; Love, Anders, and Huber 2014); and R base for metabolomics. Statistical testing to compute *p*-values using two-tailed unpaired *t*-test was followed by estimation of the false discovery rate control by computing *q*-values using Storey test (Storey et al. 2020; Pimentel et al. 2017) or *p*-adjusted values using Benjamini-Hochberg procedure-based computation (Benjamini and Hochberg 1995; Wright and Paul Wright 1992; Benjamini and Yekutieli 2001). Nearly all of such data are presented as bars or symbols showing fold change compared to control, and measurements that had *q*-value, *p*-adjusted value, or *p*-value below an indicated threshold are highlighted with colours (for example, Figure 20). In several datasets, the effect chance appeared to be less than 10%. The methods to correct for the multiplicity of comparisons have low power on such datasets, increasing the proportion of the false-negative results (Krzywinski and Altman 2014). Thus, *p*-values were used in this comparison, where p-value < 0.01 were considered as statistically significant. This was applied to the following datasets: metabolomics, 2.3 months old TwKO^{astro}; RNA sequencing, Cox10KO^{astro} and TwKO^{neuro} (for example, Figure 25).

Statistical analyses were performed using Prism 8, R, and Excel (Microsoft) software; graphs were made using Prism 8; heat maps were generated using R from scaled reads per base (Z. Gu, Eils, and Schlesner 2016); PCA and volcano plots were generated using Sleuth (Pimentel et al. 2017). Body weight progression data (from (II) and unpublished data) are plotted as mean with standard deviation (SD), and sample sizes are indicated in the legends. For a comparison of distributions of length of cilia, Kolmogorov-Smirnov test was performed using Prism 8. The morphology of cilia (Figure 35C) is plotted as average per genotype; average values for individual mice can be found in (III). Information about other statistical tests and data plotting can be found in the figure legends and described in (I-III).

5 RESULTS

5.1 Cell-specific mitochondrial dysfunction leads to disparate brain pathologies (I-III, unpublished data)

To investigate the pathogenesis arising from mitochondrial dysfunction in the brain, we disrupted mitochondrial function in neurons or astrocytes using genetically modified mice (I). First, we induced conditional knockout of *Twnk* (TwKO), which encodes mitochondrial DNA helicase Twinkle. TwKO was induced in astrocytes (TwKO^{astro}) or neurons (TwKO^{neuro}) in the mouse brain (Figure 15, see 4.1). The ultimate consequence of Twinkle depletion is loss of mtDNA replication, resulting in decreased copy number of the mitochondrial genome. This leads to depletion of mitochondrial transcripts, including those encoding essential subunits of OXPHOS complex I, III, IV and V (Figure 15, see also Figure 9). We also generated mice with an astrocytic knockout of *Cox10*, a gene encoding an assembly factor of the Complex IV, COX10 (Cox10KO^{astro}, Figure 15, see 4.1). As discussed in 4.1, Cre expression in these models starts postnatally and does not target embryonic stem cells.

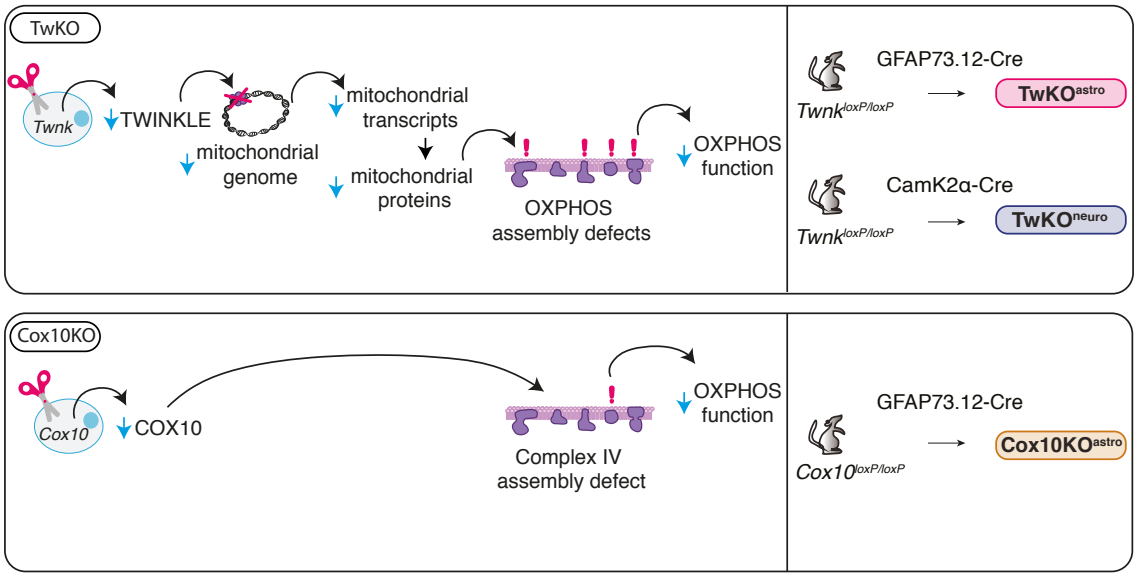

Figure 15. Schematic of the mouse models used in the research presented in this thesis (modified from (II) and unpublished). Conditional knockout of *Twnk* was induced in postnatal mouse brain astrocytes (TwKO^{astro}) or neurons (TwKO^{neuro}), and conditional knockout of *Cox10* was induced in postnatal mouse brain astrocytes (Cox10KO^{astro}). Cre expression starts postnatally in all mouse models (see 4.1).

TwKO^{astro} and TwKO^{neuro} mice were born in expected ratios with Ctrl mice, had normal postnatal development and the macroscopic appearance of the brain (I). TwKO^{astro} mice manifested with progressive body weight loss starting from 2-3 months of age, coupled with progressive muscle weakness and kyphosis (I). The maximum lifespan of TwKO^{astro} mice was 7-8 months (I). TwKO^{neuro} mice were asymptomatic before sudden deterioration at the age of 7.5-9.5 months, when mice manifested with a rapid body weight loss and a behavioral phenotype with signs of severe distress (I). This manifestation over the course of several days was followed

by mouse death (I). Since Cre recombinase is also expressed in peripheral tissues (see 4.2), Twinkle depletion in other organs can contribute to morbidity phenotypes. The research presented in this thesis was focused on phenotypes within the brain of investigated models.

In the research presented in this thesis, the preterminal stage of the phenotype development is defined at 5-5.5 months for TwKO^{astro} mice (the age when the body weight loss reaches the humane endpoint) and at 7.5-8 months for TwKO^{neuro} (the age close to sudden deterioration and death of these mice).

5.1.1 *Twnk* knockout in postnatal astrocytes or neurons leads to the loss of mitochondrial gene expression

Direct investigation of the dynamics of Twinkle loss was challenged by the absence of antibodies faithfully detecting the protein or mtDNA in mouse brain sections. To confirm that *Twnk* knockout in postnatal astrocytes or neurons results in the loss of mitochondrial gene expression, we first analysed mtDNA amount and expression of OXPHOS components in cortical lysates of TwKO^{astro} and TwKO^{neuro} mice (Figure 16A-B).

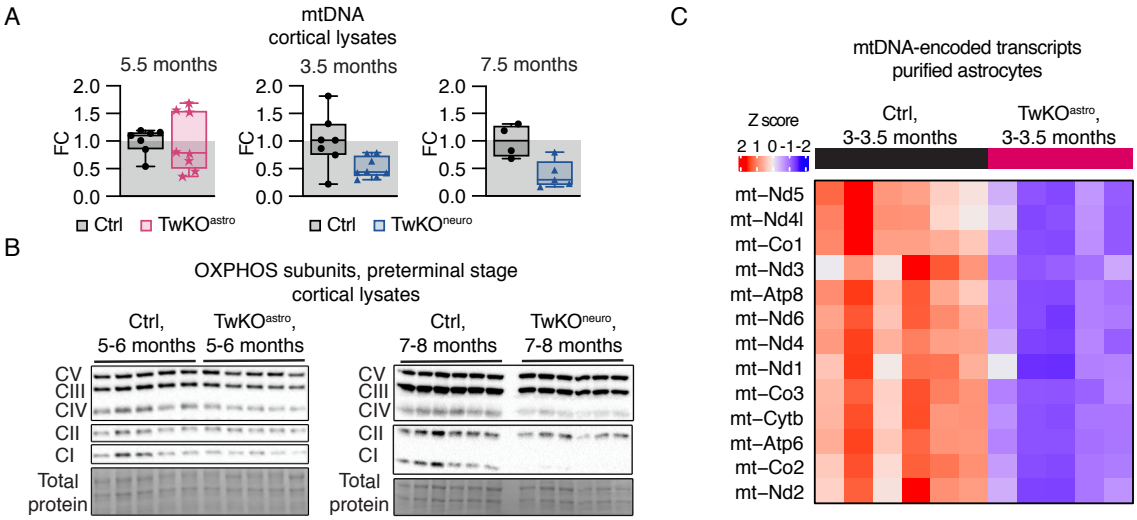

Figure 16. Mitochondrial genome expression in TwKO^{astro} and TwKO^{neuro} mice (modified from (I-III)). **(A)** mtDNA amount in cortical lysates, measured using qPCR. FC is a fold change ratio, relative to the corresponding Ctrl mice. Box and whiskers plots: the box extends from the 25th to 75th percentiles; whiskers show all points minimum to maximum; symbols represent biological replicates. **(B)** Immunoblot analysis of protein subunits of OXPHOS complexes in cortical lysates. **(C)** Levels of mtDNA-encoded transcripts measured using RNA sequencing in astrocytes purified from mouse brain cortexes (Ctrl n=6, TwKO^{astro} n=5).

At the preterminal stage, mtDNA and OXPHOS deficiency was evident in TwKO^{neuro} but not in TwKO^{astro} cortical lysates (Figure 16A-B). This might reflect either the more rapid dynamics of molecular events leading to mtDNA depletion upon *Twnk* knockout in neurons compared to astrocytes, or the sensitivity of assays to detect cell-specific loss of the mitochondrial content from cortical lysates. Specifically, if the mtDNA copy number and expression of OXPHOS components is lower in astrocytes compared to neurons and constitutes a smaller proportion of total mitochondrial content in the tissue, loss of the

mitochondrial genome in astrocytes would result in less profound changes. Consequently, we analysed the expression of mitochondrially encoded genes in astrocytes purified from TwKO^{astro} mice (Figure 16C, see 4.3). The levels of all mitochondrial transcripts were markedly decreased in astrocytes of TwKO^{astro} compared to Ctrl mice already at the age of 3-3.5 months (Figure 16C).

Collectively, this shows that postnatal knockout of *Twnk* resulted in decreased mitochondrial gene expression in both neurons and astrocytes. In both models, this loss was apparent already at 3-3.5 months, which was the earliest time point analysed (Figure 16).

5.1.2 *Twnk* knockout has different effects on mitochondrial ultrastructure in astrocytes compared to neurons

Next, we investigated the ultrastructure of mitochondria in TwKO^{astro} and TwKO^{neuro} mice. At the preterminal stage of TwKO^{neuro}, we found swollen mitochondria with tubular cristae (Figure 17). Similar ultrastructural abnormalities are observed upon the loss of mitochondrial translation in various cell types (Richter et al. 2015, 2019). In the TwKO^{astro} cortex, we first investigated mice at 4 months of age. We also found swollen mitochondria with tubular cristae, some of which were larger than any mitochondria in TwKO^{neuro} or Ctrl mice (Figure 17). Some mitochondria in TwKO^{astro} were donut-shaped, where cytoplasmic material was apparently observed in the middle of a mitochondrion (Figure 17). At the preterminal stage, we observed a large number of swollen mitochondria with electron-light areas in TwKO^{astro} (Figure 17). Throughout this thesis, these electron-light areas, as well as vacant areas observed with light microscopy are referred to as vacuoles (see also 5.1.5). We also observed intracellular membranous whirls in TwKO^{astro} mice at all analysed ages (4-8 months old mice) (Figure 17). Investigation of mitochondria at the terminal stage of TwKO^{astro} phenotype progression was challenged by the fact that there were large areas consisting mainly of the electron-light oval-shaped areas (see Figure 22). In summary, knockout of *Twnk* in both neurons and astrocytes led to mitochondrial swelling, but upon astrocytic knockout mitochondria were larger and showed progressive accumulation of electron-light areas.

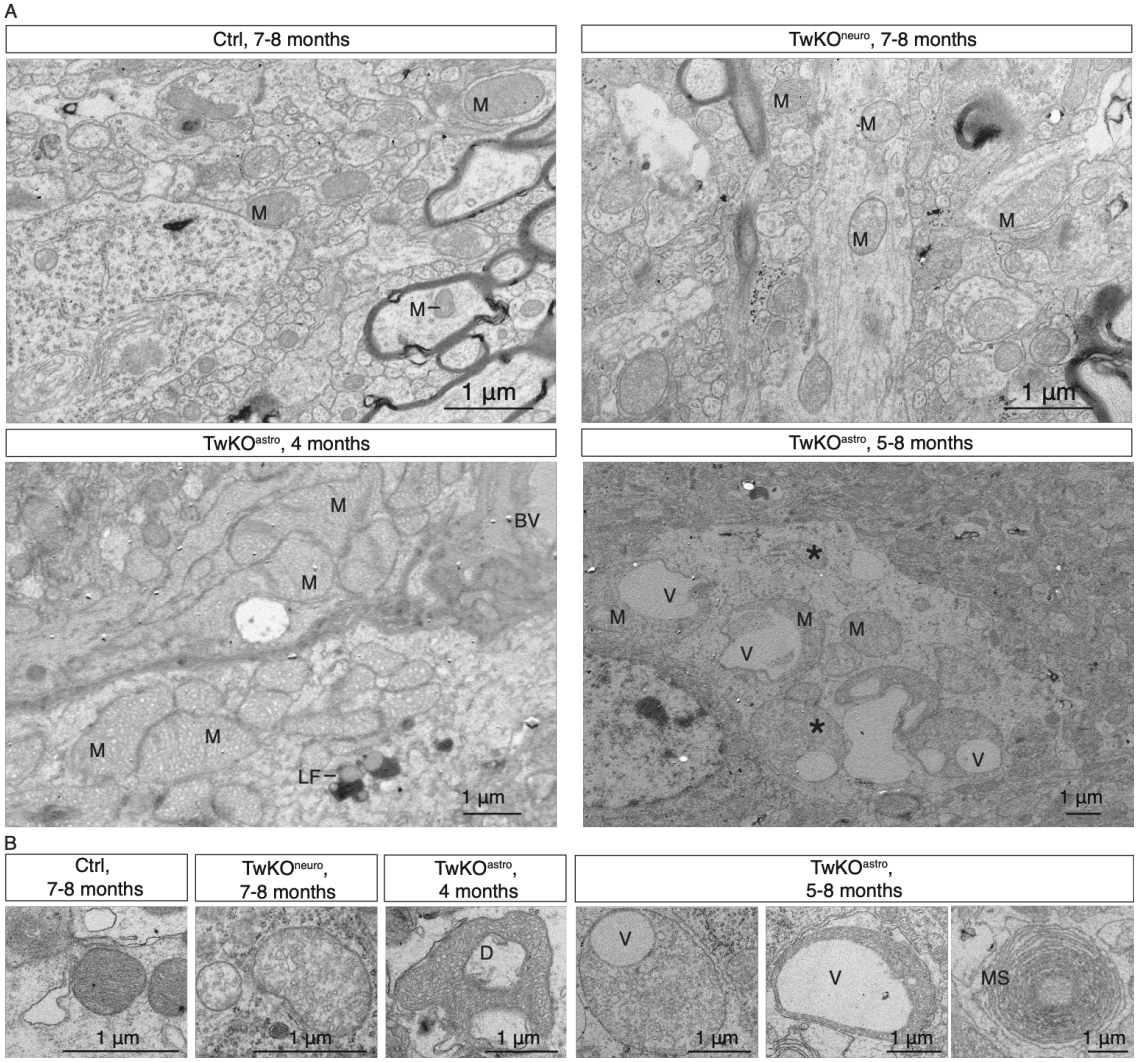

Figure 17. Transmission electron microscopy, mouse brain cortex (modified from (I) and unpublished data) **(A)** Mitochondria in the cellular context. **(B)** Mitochondria. M = a mitochondrion; V = vacuole; D = a donut-shaped mitochondrion; MS = membranous whirls; BV = blood vessel; LF = lipofuscin (see also 5.4.2.3). Asterisk in (A) indicates mitochondria that are also shown in (B) at a higher magnification. For the electron microscopy microphotograph of TwKO^{astro}, 4 months, panel (A), note that a different crop of the same image is shown also in Figure 32.

5.1.3 *Twnk* knockout in neurons leads to late-onset cell degeneration

To investigate the consequences of Twinkle loss for neuronal fitness, we used immunofluorescence against neuronal nuclear marker NeuN (Hexaribonucleotide Binding Protein-3) and against neuron-specific cytoskeletal protein MAP2 (microtubule-associated protein 2, which localises to neuronal dendrites. At the preterminal stage of TwKO^{neuro} mice, we observed profound cell degeneration, which was especially prominent in the hippocampal pyramidal neuron of CA1 hippocampal area (Figure 18). The level of neurodegeneration differed profoundly between individual mice of the same age, as some mice displayed almost complete degeneration of the CA1 area, while some did not have any signs of degeneration (I). This might indicate that neurodegeneration occured in an acute manner. We also analysed an earlier time point of six months of age and did not observe any signs of neurodegeneration (I), which further supports the conclusion that neurodegeneration was late-onset and acute. Note that the mtDNA amount was decreased in TwKO^{neuro} cortical lysates already at the age of 3.5 months (Figure 18). This acute *en mass* neurodegeneration might be causative of the sudden deterioration of TwKO^{neuro} mice observed several days before mouse death (see 5.1), but this was not specifically investigated.

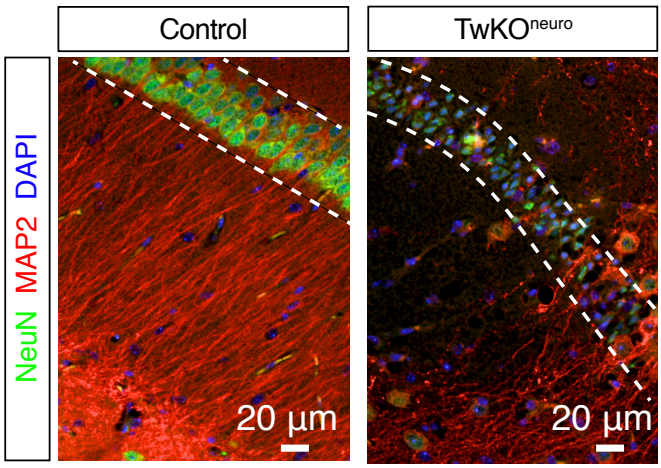

Figure 18. The CA1 area of the hippocampus, dashed lines mark the layer with neuronal soma (modified from (I)). Immunofluorescence, epifluorescence images. Imaging by Dmitri Chilov.

5.1.4 *Twnk* knockout in astrocytes leads to reactive astrogliosis

To investigate the consequences of Twinkle loss for astrocyte fitness, we used immunostainings against 10-formyltetrahydrofolate dehydrogenase (ALDH1L1), glutamine synthetase (GS), and GFAP, as well as analysed mRNA expression of astrocyte-enriched genes in TwKO^{astro} cortical lysates (Figure 19). ALDH1L1 is a specific astrocyte marker in the mouse brain cortex, whereas GS is also expressed by NG2^{+} cells (Cahoy et al. 2008; Xin et al. 2019). The number of cells positive for ALDH1L1 or GS was not decreased in the cortex of TwKO^{astro} mice at the preterminal stage (Figure 19). The number of GFAP-positive cells was drastically increased, and astrocytes displayed a typical appearance for reactive astrogliosis with thickened GFAP-positive processes (Figure 19, see also 2.2). The expression of reactive gliosis markers *Gfap* and *Vim* was increased in TwKO^{astro} mice at mRNA level, whereas the expression of several other astrocyte-enriched transcripts was unchanged (Figure 19). Together, this indicates that astrocytes survive through mitochondrial genome depletion, and display upregulated expression of intermediate filaments.

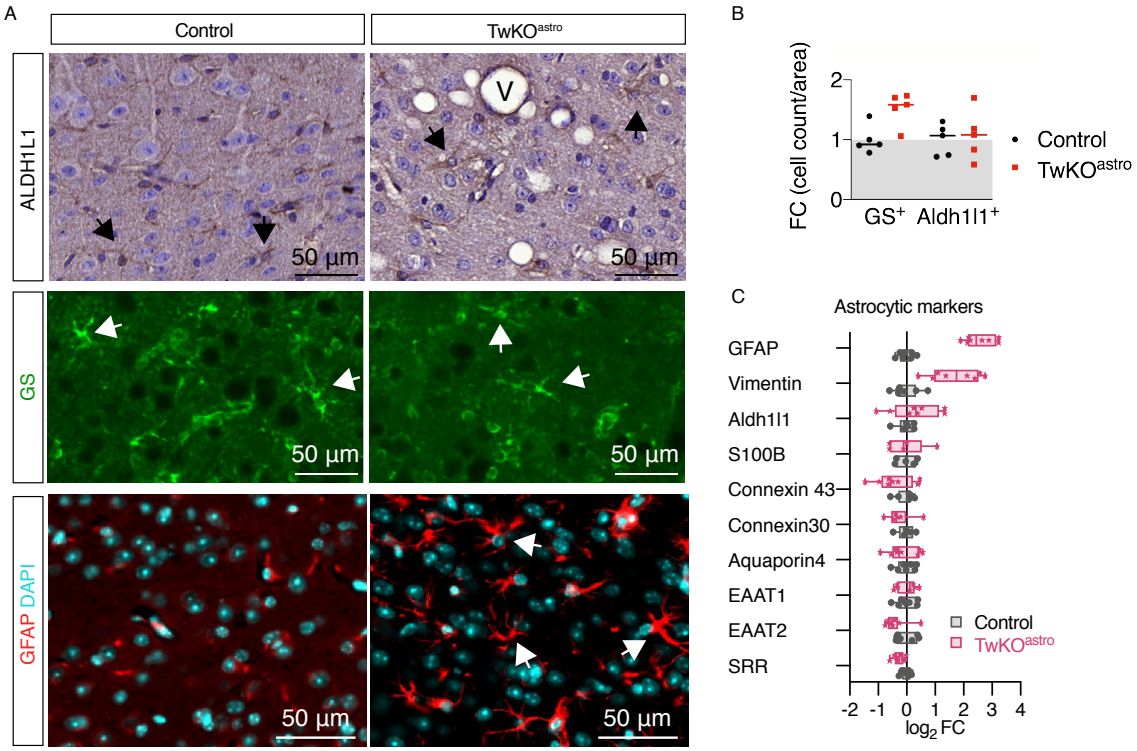

Figure 19. Expression of astrocyte-enriched genes, 5-6 months of age (modified from (I) and unpublished data). **(A)** Immunostainings, mouse brain cortex. Arrows points to cells positive for the staining of interest. V = vacuole. **(B)** Cell count, quantification of immunostainings, symbols represent an average per mouse, calculated from several sections and fields of view per mouse. **(C)** RT-qPCR, protein names are indicated. Box and whiskers plots: the box extends from the 25th to 75th percentiles; whiskers show all points minimum to maximum; symbols indicate individual mice. FC is a fold change ratio, relative to the corresponding Ctrl mice. GFAP and GS are immunofluorescence, epifluorescence images. GS panel imaging and quantification is by Gabrielle Capin.

Next, we analysed expression of reactive astrogliosis markers in astrocytes purified from TwKO^{astro} mice (Figure 20, 4.3). For this, we curated a list of genes recognised as reactive astrogliosis markers (based on (Liddelow et al. 2017; Zamanian et al. 2012; Escartin et al. 2021), see 2.2.1.2-2.2.1.3). Expression of 19 out of 58 of these genes was increased, and expression of three genes was decreased (Figure 20). In terms of function, expression of intermediate filaments and of immune-related genes was most robustly upregulated (Figure 20). Together with immunostainings (Figure 19), this indicates that reactive astrogliosis occurs in astrocytes of TwKO^{astro} mice.

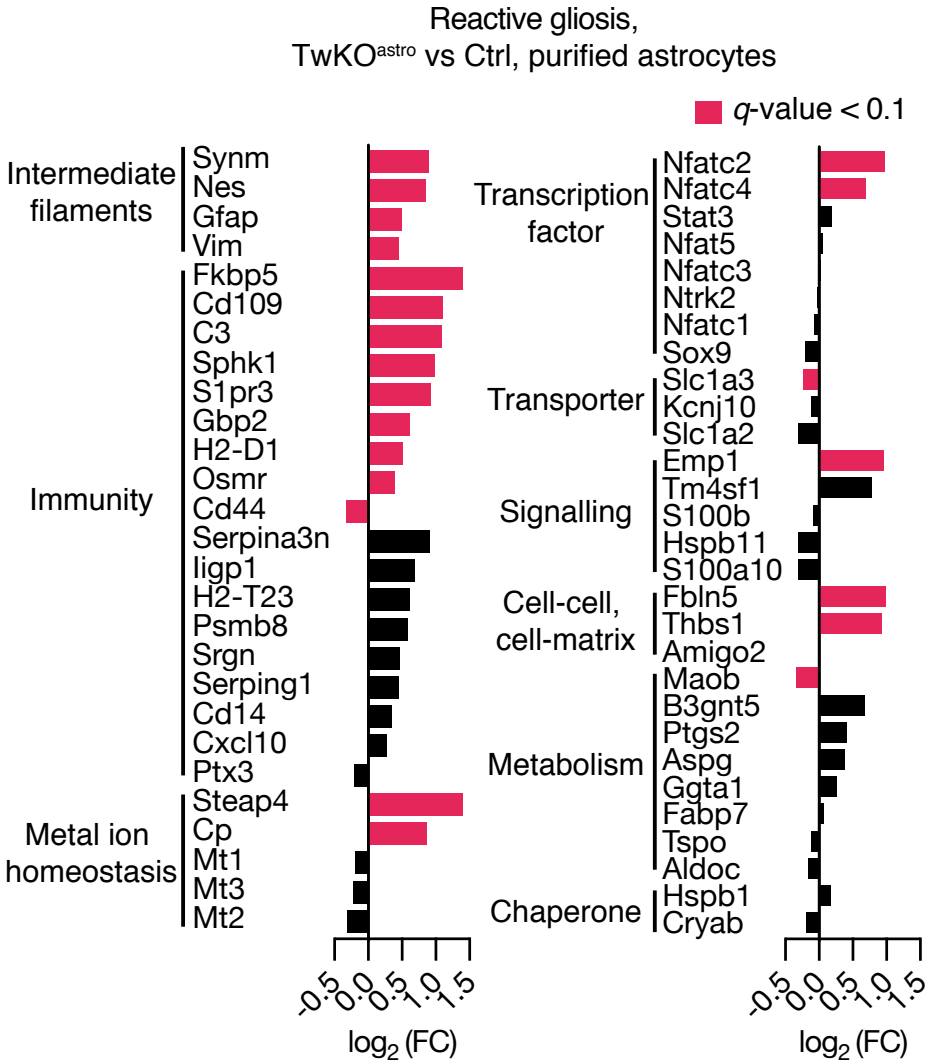

Figure 20. The expression of reactive gliosis markers, measured using RNA sequencing, astrocytes purified from brain cortexes of 3-3.5 months old mice (from (III)). FC is a fold change ratio, relative to the corresponding Ctrl mice.

Next, we aimed to determine whether GFAP protein level was upregulated cell-autonomously upon *Twnk* knockout, or if this was a response of cells not targeted by Cre expression. To do so, we analysed enzymatic activity of OXPHOS complexes on unfixed frozen brain sections of TwKO^{astro} mice using a colorimetric assay (Figure 21). The assembly of Complex IV requires subunits encoded in both the nuclear and the mitochondrial genome, whereas Complex II is encoded solely in the nuclear genome and thus might be assembled in the absence of mitochondrial genome expression (see 2.3.2-2.3.4). In the brain of TwKO^{astro} mice, we found cells with undetectable activity of Complex IV, in which the activity of Complex II was still preserved (Figure 21). Using immunofluorescence against a subunit of Complex II, we also found its accumulation in GFAP-positive astrocytes (Figure 21). This was especially evident in the areas with low cell density, such as in the hippocampus (Figure 21) and the pia mater (data not shown). Increased Complex II signal is consistent with the presence of enlarged mitochondria in TwKO^{astro} cortex (Figure 17). In the Ctrl brain, most of the signal in immunostaining against a subunit of Complex II was from the perinuclear mitochondrial ring of the neurons, while the signal in astrocytes was nearly absent. Similar staining patterns were also observed in immunostainings to other mitochondrial components, hindering the detailed investigation of mitochondria in astrocytes of TwKO^{astro} mice using immunofluorescence (data not shown)

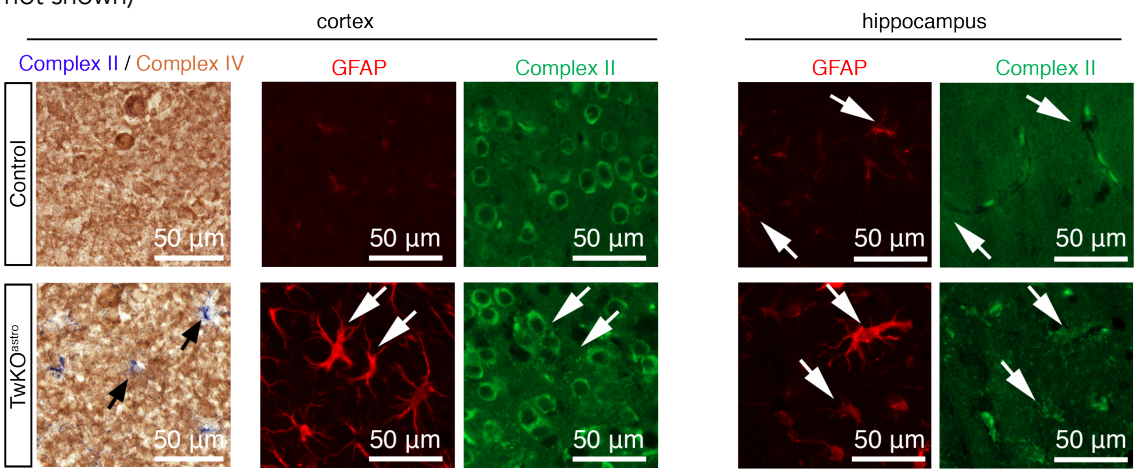

Figure 21. Colorimetric assay for the enzymatic activity of Complex II and IV, immunostainings against the subunit of Complex II (SDHA) and GFAP (modified from (I)). Preterminal stage of TwKO^{astro} mice, 5-6 months. Left panel: cortex. Right panel: hippocampus, area below CA1 neuronal layer is shown. For immunofluorescence, epifluorescence images are shown.

Together, this indicates that the upregulation of GFAP expression is cell-autonomous in astrocytes upon loss of mitochondrial gene expression. The direct investigation of Twinkle or mtDNA level in GFAP-positive astrocytes was however hindered by the absence of suitable antibodies.

5.1.5 *Twnk* knockout in astrocytes leads to spongiotic pathology

Next, we investigated if TwKO^{astro} mice manifested with histopathological abnormalities. We stained mouse brain sections after paraffin-embedded with hematoxylin and eosin. In TwKO^{astro} brain parenchyma, we observed abnormalities that looked like vacant spots of oval and round shapes, which we named vacuoles (Figure 22, see also Figure 17). The first signs of pathology appeared at the age of ~2 months as sparsely located vacuoles, progressing to mesh-like pathology at the terminal stage of 7-8 months (I). This was coinciding with reactive astrogliosis, as GFAP-positive astrocytes occurred nearby vacuolated areas (data not shown). Frequently, GFAP-positive processes of astrocytes were enwrapped around the vacuoles (Figure 22). Electron microscopy also revealed the presence of electron-light areas of oval and round shapes, which sometimes contained debris (Figure 22).

This pathology was similar to histopathological observations in several human mitochondrial diseases (Nolte et al. 2013; Sofou et al. 2012). Following the nomenclature accepted for these diseases, we named the brain pathology of TwKO^{astro} mice as spongiotic encephalopathy (I).

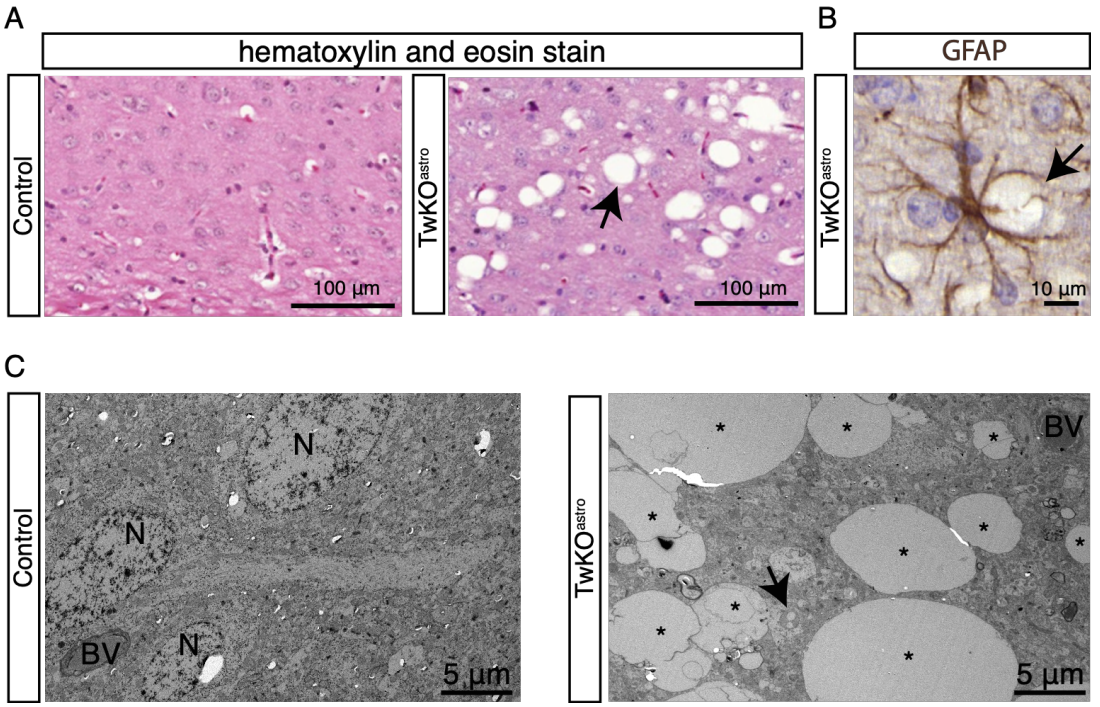

Figure 22. Brain pathology in TwKO^{astro} mice, five to eight months of age (modified from (I) and unpublished data). (A) Hematoxylin and eosin stain, an arrow points to a vacuole. (B) Immunostaining against GFAP, an arrow points to a vacuole. (C) Transmission electron microscopy, cortex. * indicate vacuoles in TwKO^{astro}. An arrow points to a swollen mitochondrion harboring electron-light areas (see also Figure 17). N = nucleus, BV = blood vessel.

5.1.6 *Cox10* knockout in astrocytes manifests similarly to Twnk knockout

Cox10KO^{astro} manifested with body weight loss (Figure 23). Some mice displayed progressive body weight loss and reached humane endpoint by four months, which was sooner than TwKO^{astro} mice (Figure 23A, (I-II)). Other Cox10KO^{astro} mice lost 10-15% of the maximum body weight by the age of four months, but the weight did not decrease further up to at least seven months of age (data not shown). During the time of this thesis submission for examination, the later time points were being investigated.

To investigate brain pathology of Cox10KO^{astro} mice, we used hematoxylin and eosin stain and immunostaining against GFAP. GFAP expression was increased in Cox10KO^{astro} compared to Ctrl and Cox10KO^{astro} presented with spongiotic encephalopathy (Figure 23B-C). These findings occurred in both Cox10KO^{astro} females and males (data not shown) and were similar to TwKO^{astro} mice (Figure 19A, 22A). The severity of spongiotic encephalopathy was comparable in the cortex of these two models, although variable (data not shown). In Cox10KO^{astro} mice, cerebellum was the most affected region, and the pathology was more severe compared to TwKO^{astro} mice (Figure 23). Together, this indicates that loss of mitochondrial gene expression or Complex IV assembly in postnatal mouse astrocytes result in similar brain pathologies.

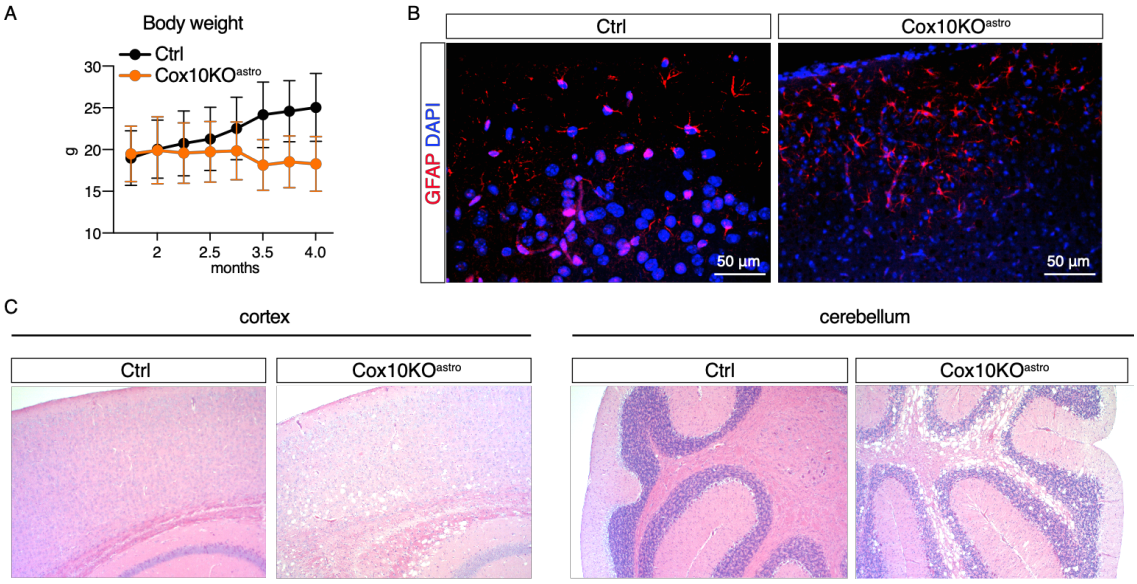

Figure 23. Cox10KO^{astro} mice, 4 months of age (unpublished data). **(A)** Body weight, male mice. Ctrl (n=6), Cox10KO^{astro} (n=6). Mean with SD is shown. **(B)** Immunostaining against GFAP, cortex. MIP, confocal images. **(C)** Hematoxylin and eosin stain.

5.2 Cell-specific mitochondrial dysfunction leads to disparate cell responses (II, unpublished data)

To investigate cell responses to mitochondrial dysfunction in the CNS, we analysed the core ISR^{mt} gene expression and metabolic signature in cortical lysates of TwKO^{astro} and TwKO^{neuro} mice ((II), see 2.3.6.3). Expression of several analysed genes was increased in TwKO^{astro} compared to Ctrl mice (Figure 24). Consistently with upregulation of mRNAs of serine biosynthesis genes, levels of serine and glycine were also upregulated in TwKO^{astro} mice (Figure 24). In contrast, only *Atf3* expression was induced in TwKO^{neuro} mice, and its expression was variable (Figure 24).

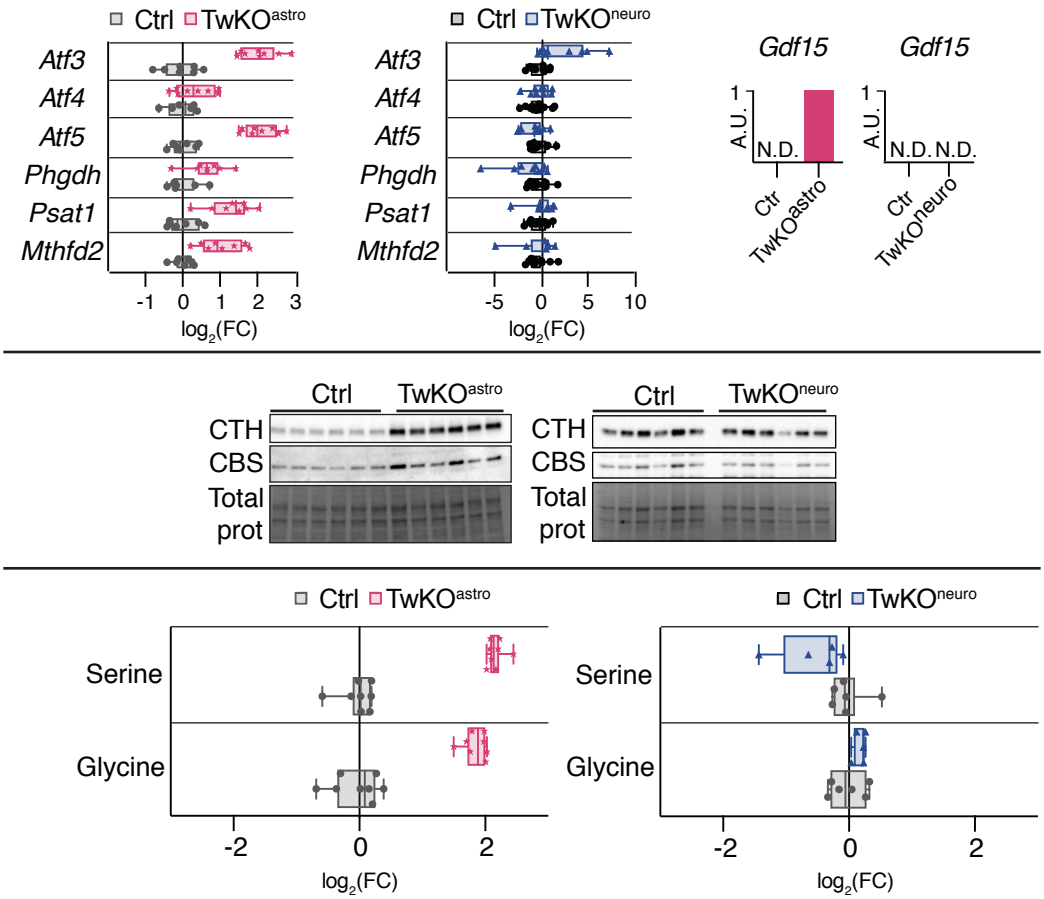

Figure 24. Integrated stress response signature in TwKO^{astro} (5.5 months old) and TwKO^{neuro} (7.5 months old) cortical lysates at the preterminal stage (modified from (II)). Top panel: mRNA levels measured with RT-qPCR. Middle panel: immunoblot analysis. Bottom panel: amino acid levels measured with targeted metabolomics. FC is a fold change ratio, relative to the corresponding Ctrl mice. Box and whiskers plots: the box extends from the 25th to 75th percentiles; whiskers show all points minimum to maximum; symbols indicate biological replicates. *Gdf15* expression was not detected in Ctrl mice, but was detected in all TwKO^{astro} mice (presented as a.u. = 1); Ctrl (n = 7), TwKOastro (n = 8).

To investigate the same response in Cox10KO^{astro} cortical lysates, we used RNA sequencing. In addition, the core ISR^{mt} gene expression signature, we analysed the following genes that are implicated in ISR: *Eif4ebp1* (translation initiation factor), *Mthfd1l* (folate cycle, same pathway as *Mthfd2*), *Psph* (serine biosynthesis, same pathway as *Phgdh* and *Psat1*), as well as *Trib3*, *Eprs*, *Slc7a3*, *Asns*, *Chac1*. Cox10KO^{astro} displayed upregulation of all aforementioned pathways (Figure 25). In parallel, we also performed RNA sequencing on a cohort of TwKO^{neuro} mice at the preterminal stage of the phenotype progression (mice were two weeks older compared to the cohort presented in Figure 24). *Atf3* was the only clearly induced gene (Figure 24, 25). *Gdf15* expression was variable, and in both Cox10KO^{astro} and TwKO^{neuro} groups one mouse showed a clear upregulation (Figure 25).

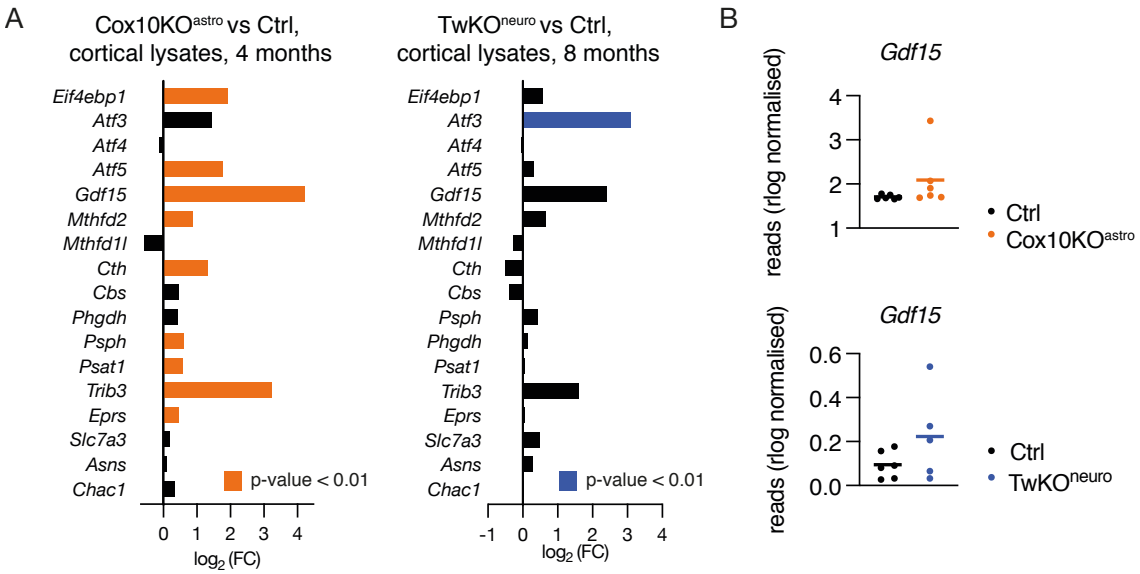

Figure 25. Integrated stress response signature in Cox10KO^{astro} (4 months old mice) and TwKO^{neuro} (8 months old mice) cortical lysates, RNA sequencing (unpublished data). **(A)** Log_2(FC) is shown. **(B)** Normalised reads are shown, symbols indicate individual mice. Cox10KO^{astro} experiment: n=6 per group, mixed sex cohort. TwKO^{neuro} experiment: Ctrl (n=6), TwKO^{neuro} (n=5), male mice were analysed. FC is a fold change ratio, relative to the corresponding Ctrl mice.

5.3 Brain pathology caused by *Twnk* knockout in astrocytes is refractory to treatment with rapamycin or a ketogenic diet (II)

As discussed in 2.3.6.4, administration of the mTorc1 inhibitor rapamycin modulated morbidity of several mouse models of mitochondrial dysfunction, and ketogenic diets are administered on the elective basis to patients with drug-resistant epilepsy. Here, we tested the efficacy of these treatment approaches to modulate brain pathology of TwKO^{astro} mice (II). We started both treatments at the presymptomatic age of 1-1.5 months (Figure 26), when we did not observe signs of reactive astrogliosis or spongiotic encephalopathy (I). Both treatments resulted in accelerated body weight loss in TwKO^{astro} mice, and were terminated once the humane endpoint of the body weight loss was reached (Figure 26).

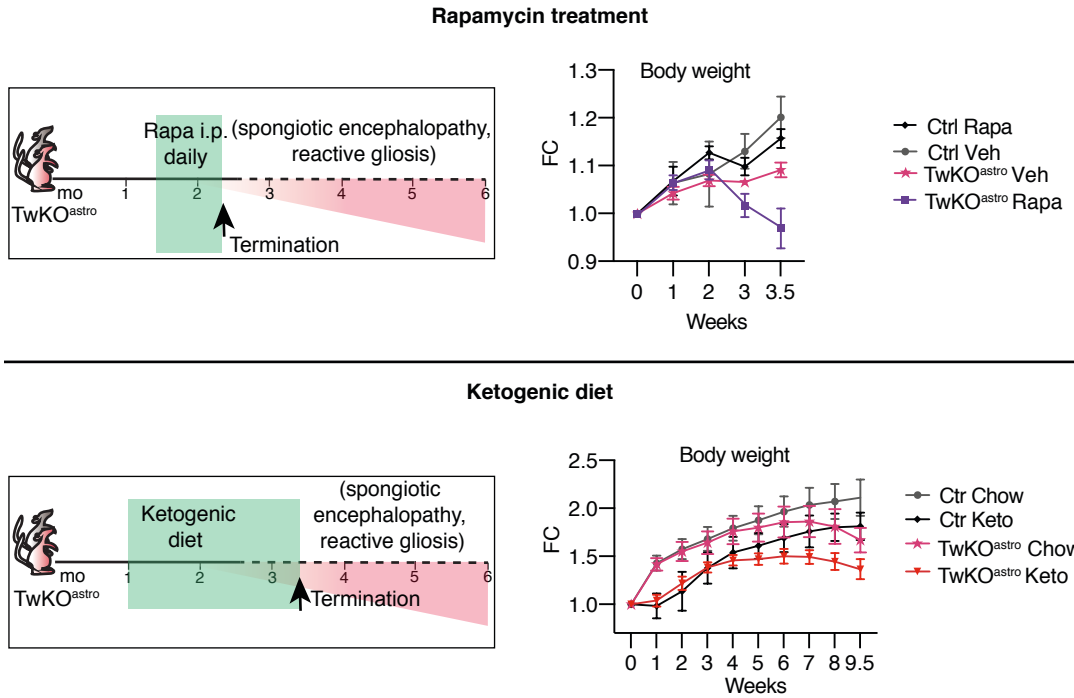

Figure 26. Schematic of the treatments and body weight progression (from (II)). In schematics, the green area shows a treatment period and the pink area represents symptom progression in untreated TwKO^{astro} mice. Body weight graphs, mean with SD is shown. Ctrl Veh (n=9), Ctrl Rapa (n=9), TwKO^{astro} Veh (n=9), TwKO^{astro} Rapa (n=9). Ctrl Chow (n=10), Ctrl Keto (n=7), TwKO^{astro} Chow (n=9), TwKO^{astro} Keto (n=8). FC is a fold change ratio, relative to the body weight at treatment initiation (zero time point). Veh = vehicle; rapa = rapamycin; chow = chow diet; keto = ketogenic diet; i.p. = intraperitoneal injection.

Rapamycin did not have an evident effect on spongiotic pathology or GFAP expression level in TwKO^{astro} mice, while the ketogenic diet exacerbated these phenotypes (Figure 27). Consequently, we concluded that neither rapamycin nor ketogenic were effective as a treatment strategy for the brain pathology that occurs due to mitochondrial dysfunction in astrocytes.

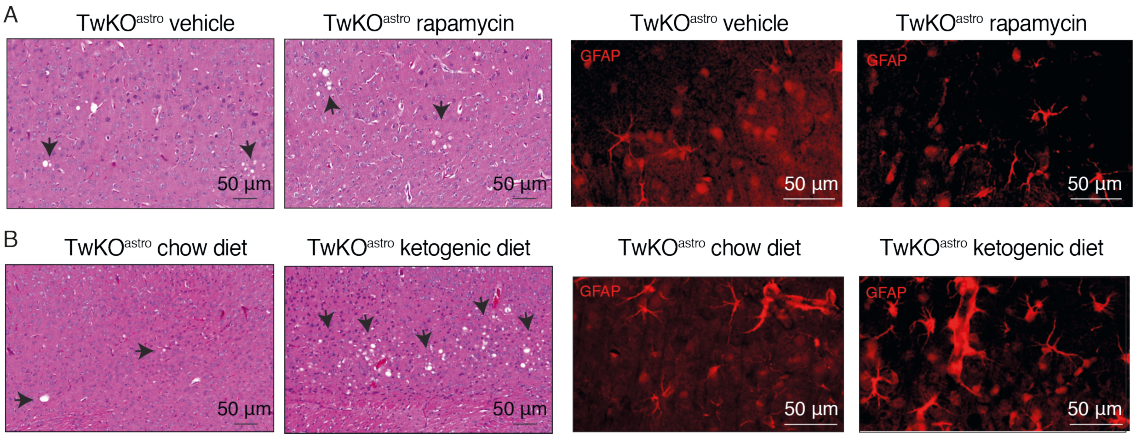

Figure 27. Brain pathology of TwKO^{astro} mice after rapamycin (A) or a ketogenic diet (B) administration, cortex. Hematoxylin and eosin stain; immunostaining against GFAP (epifluorescence). Arrowheads point to vacuoles.

5.4 Astrocyte responses to *Twnk* knockout (I-III, unpublished data)

In the research presented in the first part of this thesis (I-II), we investigated consequences of mitochondrial dysfunction in astrocytes using histopathological characterisation and analysed known cell responses to stress, namely reactive astrogliosis and integrated stress responses. To further investigate astrocyte responses to *Twnk* loss, we analysed the transcriptome of astrocytes purified from 3-3.5 months old TwKO^{astro} and Ctrl mice using RNA sequencing (see 4.2). Finally, we followed up key findings by additional experimentation (III).

5.4.1 *Twnk* knockout induces transcriptional responses in astrocytes

The transcriptome of astrocytes was globally affected by the knockout of *Twnk*, as the principal component analysis suggested marked differences between the genotypes (Figure 28A). Expression levels of 1131 genes were upregulated and of 408 were downregulated in astrocytes purified from TwKO^{astro} (Figure 28B). Among the most downregulated transcripts were mtDNA-encoded transcripts (Figure 28B, see also Figure 16C), and among the most upregulated transcripts were ISR-associated genes (Figure 28B).

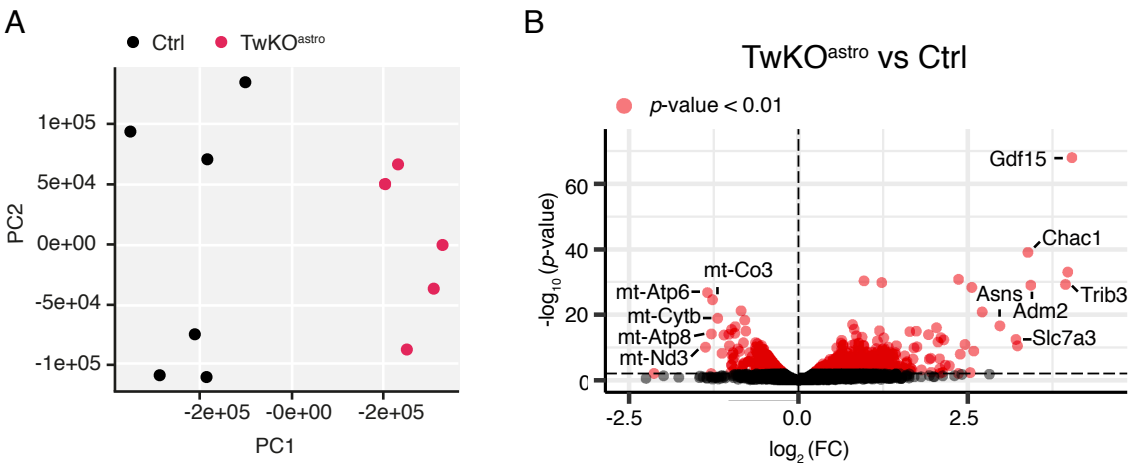

Figure 28. RNA sequencing, astrocytes sorted from brain cortexes of 3-3.5 months old mice. **(A):** Principal component analysis. **(B):** Volcano plot, all identified transcripts. Most downregulated genes that are encoded in the mitochondrial genome and most upregulated genes that are markers of the ISR are denoted with gene symbols. FC is a fold change ratio, relative to the corresponding Ctrl mice.

5.4.2 *Twnk* knockout in astrocytes affects brain lipid homeostasis

To investigate which processes were downregulated in astrocytes sorted from TwKO^{astro} brain, we used gene ontology analysis of genes, expression of which was decreased in our dataset (III). We discovered that four out of the top ten downregulated pathways were related to lipid biosynthesis (Figure 29).

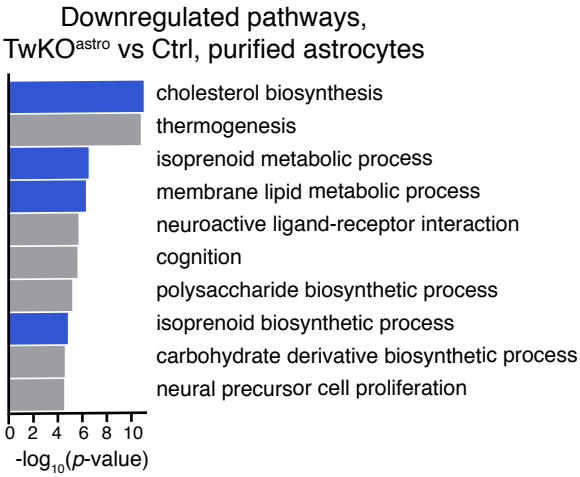

Figure 29. Gene ontology analysis of genes, expression of which was decreased in TwKO^{astro} compared to Ctrl mice (from (III)). RNA sequencing, astrocytes sorted from brain cortexes of 3-3.5 months old mice. Blue indicates pathways related to lipid metabolism

5.4.2.1 Brain lipid composition is affected by Twnk knockout in astrocytes

To investigate levels of lipid species in TwKO^{astro} compared to Ctrl mice, we used untargeted metabolomics (II). At the age of 2.3 months, the levels of only three lipid metabolites were changed (two ceramides and a cholesteryl ester) (Figure 30). At 3.2 months, levels of 106 out of 172 identified lipids and lipid-like molecules were changed in TwKO^{astro} cortical lysates compared to Ctrl mice (Figure 30). Levels of most metabolites belonging to subclasses of steroids and derivatives, fatty acids and other acyls, as well as prenol lipids were lower in TwKO^{astro} compared to Ctrl mice (Figure 30). This is consistent with downregulated expression of lipid biosynthesis pathways in astrocytes purified from TwKO^{astro} mice at 3-3.5 months of age (Figure 29). Levels of many glycerophospholipids were increased or decreased, and levels of several sphingolipids, as well as three identified glycerolipids, were also changed (Figure 30). In summary, we found that downregulation of lipid biosynthesis at the transcriptional level in astrocytes sorted from TwKO^{astro} mice is coupled with dysregulation of the lipid composition of the brain (Figures 29-30).

In contrast, we did not observe similar changes in the levels of lipid metabolites in the brain of TwKO^{neuro} mice (Figure 30). At 3.5 months of age, levels of only two out of 172 identified lipid metabolites were changed (Figure 30). At the preterminal stage of 7.5 months, levels of nine lipid metabolites were changed in TwKO^{neuro} compared to Ctrl mice (Figure 30).

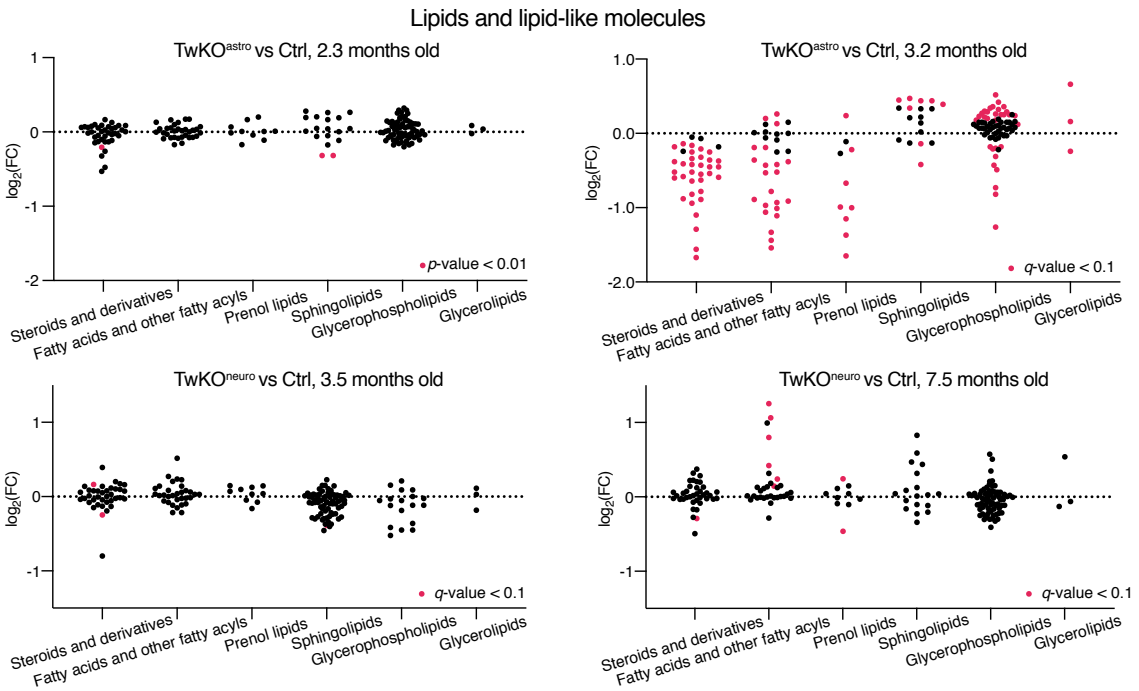

Figure 30. Lipids and lipid-like molecules, untargeted metabolomics (modified from (II-III)). FC is a fold change ratio, relative to the corresponding Ctrl mice.

5.4.2.2 Coenzyme Q biosynthesis is affected by *Twnk* knockout in astrocytes.

Ubiquinone-1 (also known as coenzyme Q1) and ubiquinone-2 (also known as coenzyme Q2) were among prenol lipids depleted in TwKO^{astro} compared to Ctrl mice (Figure 31A). These metabolites are intermediates in the synthesis of coenzyme Q, thus their depleted levels may suggest a secondary coenzyme Q deficiency (electron carrier function of coenzyme Q in the respiratory chain is discussed in 2.3.2). In turn, levels of both metabolites in TwKO^{neuro} were comparable to Ctrl mice (Figure 31A).

Next, we investigated whether coenzyme Q biosynthesis was downregulated at the transcriptional level in astrocytes purified from TwKO^{astro} mice. Coenzyme Q biosynthesis occurs in mitochondria (catalyzed by enzymes encoded by *Coq1-Coq11* genes) (Figure 31B). Coenzyme Q biosynthesis requires metabolites provided by the mevalonate pathway, which is localised outside mitochondria (Figure 31B). The expression of mevalonate pathways enzymes was downregulated in astrocytes purified from TwKO^{astro} mice (Figure 31C). Of *Coq* genes, only *Coq9* was downregulated (Figure 31C). This suggests that coenzyme Q biosynthesis might be impaired via decreased production of its precursors in the mevalonate pathway. Other metabolites that are produced in mevalonate pathway include precursors of cholesterol and steroid hormones, the levels of which were depleted in TwKO^{astro} brain (Figure 30).

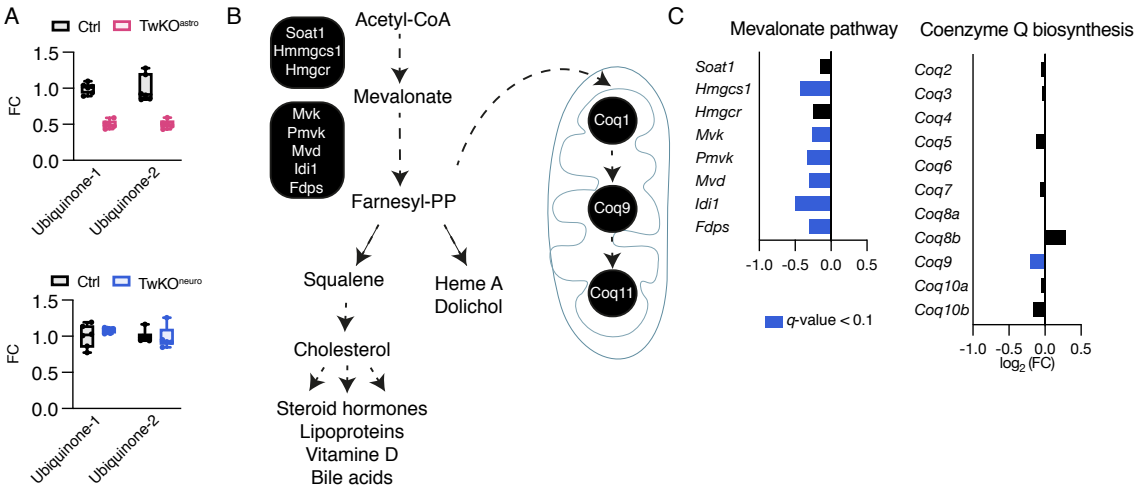

Figure 31. Coenzyme Q biosynthesis. **(A)** Untargeted metabolomics, TwKO^{astro} and the corresponding Ctrl (3.2 months old), TwKO^{neuro} and the corresponding Ctrl (7.5 months old). **(B)** Simplified schematic of the mevalonate (on the left) and coenzyme Q biosynthesis (on the right) pathways. Names of the genes encoding enzymes of the pathway are highlighted in black boxes. **(C)** Expression of genes highlighted in (B) in astrocytes sorted from TwKO^{astro} compared to Ctrl mice (RNA sequencing). FC is a fold change ratio, relative to the corresponding Ctrl mice.

5.4.2.3 *Twnk* knockout in astrocytes leads to lipid droplet accumulation in the brain

A hallmark of various brain pathologies is lipid droplet accumulation (reviewed in (Farmer et al. 2020)). We investigated whether mitochondrial dysfunction in astrocytes leading to dysregulation of lipid homeostasis was associated with lipid droplet accumulation in TwKO^{astro} mice.

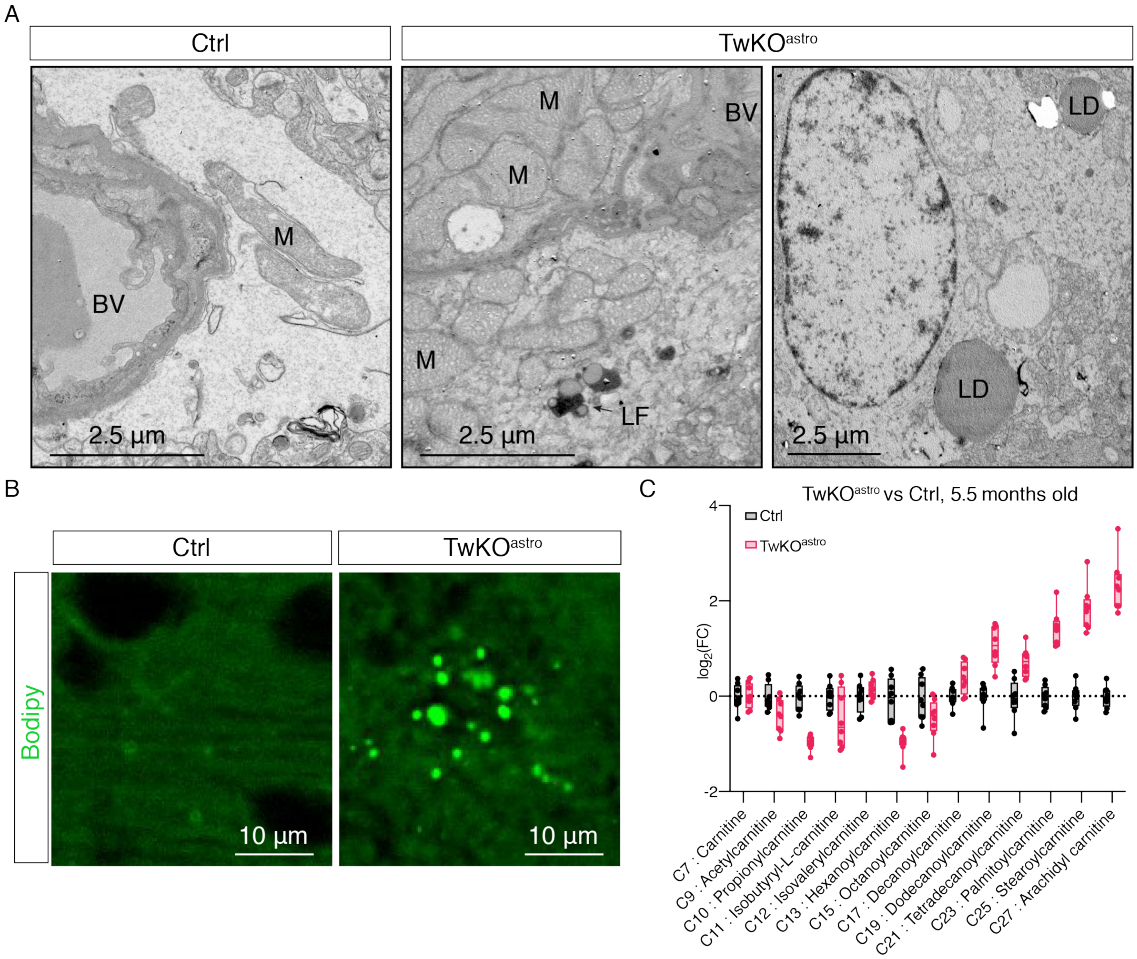

Figure 32. Lipid species at the preterminal stage of TwKO^{astro}, cortex. **(A):** Transmission electron microscopy, 4-8 months old mice (unpublished data). LD = lipid droplet, LF = lipofuscin, M = mitochondrion, BV = blood vessel. **(B):** Bodipy staining, 5.5 months old mice (unpublished data). Epifluorescence images. **(C):** Carnitine levels, 5.5 months old TwKO^{astro} mice, targeted metabolomics (modified from (II)). FC is a fold change ratio, relative to the corresponding Ctrl mice. For the electron microscopy microphotograph in the middle, note that a different crop of the same image is shown also in Figure 17.

Using transmission electron microscopy, we found accumulation of lipid droplets in the brain of 5-8 months old TwKO^{astro} mice (Figure 32A). We also observed lipofuscin in TwKO^{astro} brain (Figure 32A, 17A). Lipofuscin formations are considered as lipid-containing residues of lysosomal degradation and are often seen in brain pathologies and in ageing (Hendy 1971; Biesemeier, Schraermeyer, and Eibl 2011; Double et al. 2008). We further investigated accumulation of lipids in TwKO^{astro} brain at the preterminal stage biochemically by using a fluorescent compound bodipy, which has affinity to neutral lipids (Figure 32). In mice of both genotypes, we observed bodipy-positive filaments, which we interpreted as myelinated fibers (data not shown). In the brain of TwKO^{astro} mice, we also found accumulation of bodipy-positive particles (Figure 32B). Next, we performed targeted metabolomics on cortical lysates at the preterminal stage of TwKO^{astro} mice at 5.5 months of age (Figure 32C). In this dataset, the only

identified lipid species were carnitines. Remarkably, while the levels of several mid-chain carnitines were depleted, levels of long-chain carnitines were increased (Figure 32C; note that carnitines were not identified in the untargeted metabolomics dataset presented in Figure 30).

5.4.3 *Twnk* knockout induces a ciliogenic program in astrocytes

To investigate which processes were induced in astrocytes upon *Twnk* knockout, we used gene ontology analysis of genes, expression of which was increased in astrocytes purified from TwKO^{astro} compared to Ctrl mice (III). The top five upregulated pathways were related to cilia (Figure 33A). Cilia are microtubular organelles that face the cell surface and are classified as primary (non-motile) or motile. Primary cilia have a sensory function and compartmentalise membrane receptors for signal transduction pathways including hedgehog and G protein-coupled receptors (reviewed in (Nachury and Mick 2019)). They are present on a majority of eukaryotic cell types. In contrast, motile cilia are best known for their role in facilitating the movement of fluid at the surface of lumen-facing cell types (Olstad et al. 2019; Hoyer-Fender 2013). Astrocytes are known to harbor only a primary immotile cilium, however all ciliary pathways upregulated in TwKO^{astro} astrocytes were related to ciliary movement (Figure 33A).

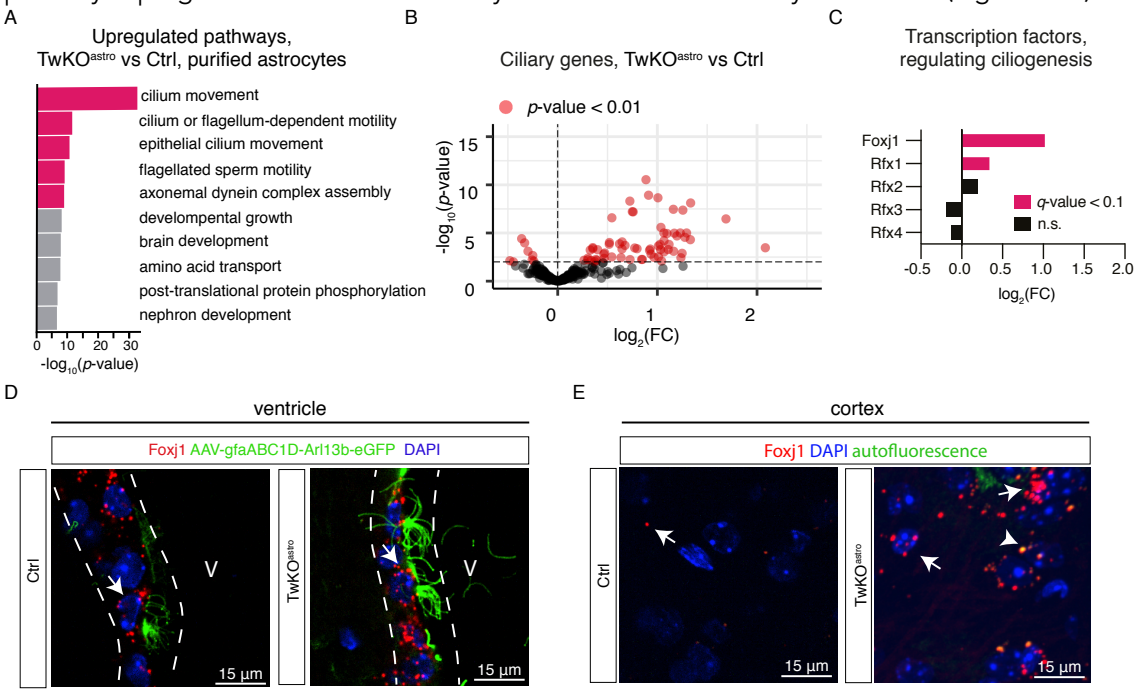

Figure 33. Ciliogenic program is induced upon Twnk knockout in astrocytes (from (III)). **(A-C):** RNA sequencing, astrocytes purified from brain cortices of 3-3.5 months old TwKO^{astro} and Ctrl mice. **(A):** gene ontology analysis of genes with increased expression **(B):** expression of ciliary genes. **(C):** expression of transcription factors, regulating ciliogenesis. **(D):** Foxj1 RNA-fluorescence *in situ* hybridization, 3.5 months old mice. Cilia are visualised by expressing an axonemal component ARL13B using an AAV vector. Dashed lines mark the ependymal cell layer, arrows show ependymal cells with Foxj1 signal. V = ventricle. **(E):** Foxj1 RNA-fluorescence *in situ* hybridization, 3.5 months old mice, cortex. Arrows show Foxj1 signal. Signal positive for both Foxj1 probe and autofluorescence was interpreted as unspecific (arrowhead). (D-E): MIP, confocal images. FC is a fold change ratio, relative to the corresponding Ctrl mice.

Next, we analysed expression levels of cilia genes in our dataset, and found that the expression of 64 out of 280 genes was upregulated (Figure 33B). In the brain, ciliogenesis and expression of ciliary genes are regulated by transcription factors of the RFX family and FOXJ1 (reviewed in (Thomas et al. 2010; Choksi et al. 2014)). Of these, the expression of the master regulator of motile ciliogenesis *Foxj1* was most upregulated (Figure 33C).

FOXJ1 is presumed to be active only in multiciliated cells harboring motile cilia, such as ependymal cells in the brain (Mukherjee, Roy, and Chakrabarti 2019; Patir et al. 2020). Astrocytes harbor a solitary immotile cilium, and are not known to express FOXJ1. To investigate the spatial *Foxj1* expression, we used RNA-fluorescence *in situ* hybridization. As expected, we detected *Foxj1* expression in a layer of multiciliated ependymal cells forming the ventricular wall (Figure 33D). In the cortex of Ctrl mice, we only observed rare *Foxj1*-positive punctae (Figure 33E). Strikingly, we observed marked upregulation of *Foxj1* expression in the cerebral cortices of TwKO^{astro} mice (Figure 33E).

5.4.3.1 *Twnk* knockout results in anomalous expression of motile cilia components in astrocytes

Next, we investigated expression levels of motile cilia components in astrocytes purified from TwKO^{astro} compared to Ctrl mice. Axonemes of both primary and motile cilia comprise 9 outer doublet microtubules; motile cilia also harbor a set of distinct components essential for its movement (Figure 34A). Expression of genes that compose all key structures specific to motile cilia was induced in TwKO^{astro} compared to Ctrl (Figure 34B). These included dynein arms and cytoplasmic factors for their assembly, nexin-dynein regulatory complex (nexin link) that is located between microtubule doublets, central pair that is an additional microtubule doublet, radial spokes that protrude towards the central pair, and other factors involved in assembly and motility of the axoneme (Figure 34B). Together, induced expression of *Foxj1* and motile components suggest activation of motile ciliogenesis program in TwKO^{astro} (Figure 33-34).

In the brain, motile ciliogenesis is tightly linked to differentiation of multiciliated ependymal cells (Avasthi and Marshall 2012). The principal events in this pathway include cell cycle exit, centriole amplification, orientation and docking of basal bodies to an apical membrane, and the axoneme extension (Figure 34C). Expression level of key regulators of these steps (*Trp73*, *Cdkn1a*, *Myb*, *Foxj1*, *Ank3*, *Six*) was increased in our dataset (Figure 34D) (Nemajerova et al. 2016; Marques et al. 2019; Kyrousi, Lygerou, and Taraviras 2017)).

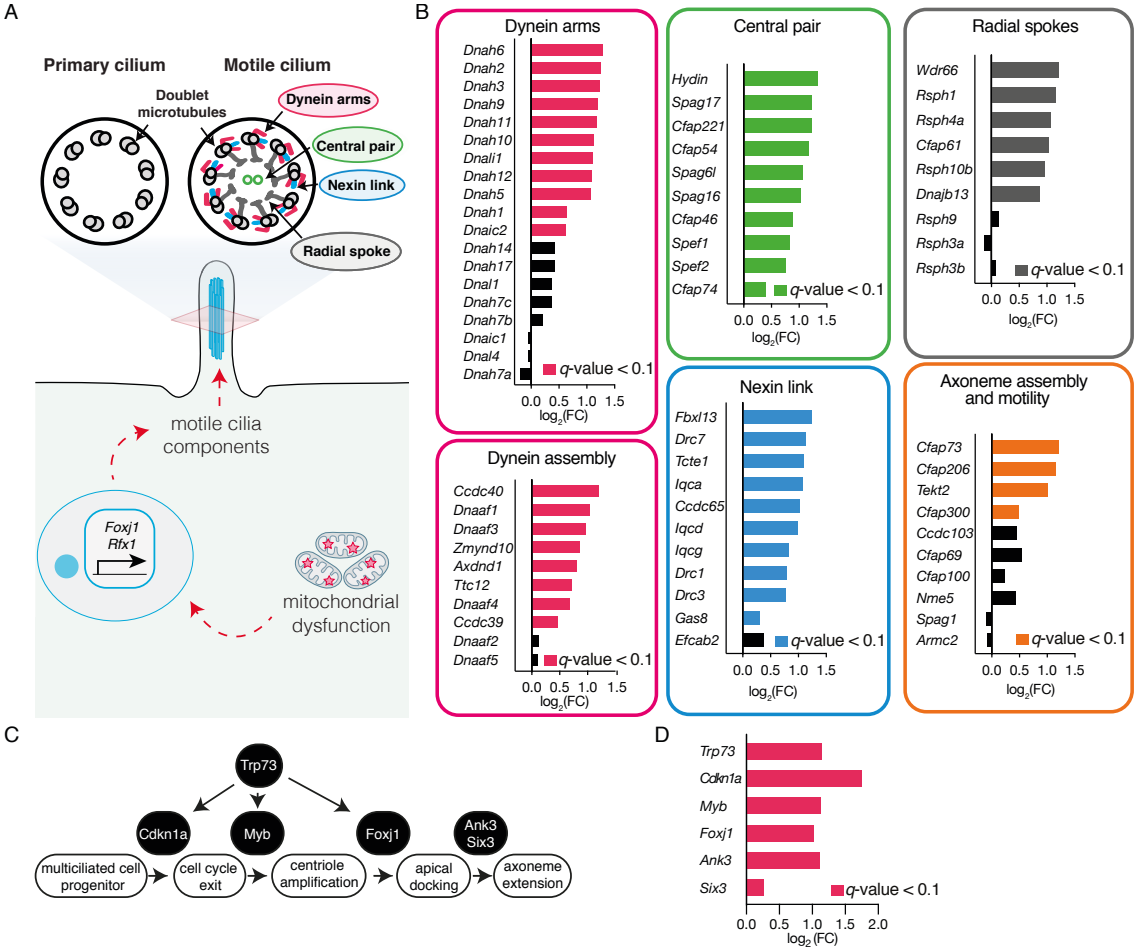

Figure 34. Expression of motile cilia components in astrocytes of TwKO^{astro} mice (from (III)). **(A):** Schematic of the cilia cross section. Axonemes of both primary and motile cilia comprise nine doublet microtubules. Motile cilia also harbor components not present in primary cilia. **(B):** Expression of structural components specific to motile cilia and their assembly factors in astrocytes sorted from TwKO^{astro} mice compared to Ctrl. **(C):** Schematic of the key steps of multiciliated cell differentiation from the cell cycle exit to motile cilia formation. Gene symbols indicate regulators of these events. **(D):** Expression of genes highlighted in (C) in astrocytes purified from TwKO^{astro} mice compared to Ctrl mice. FC is a fold change ratio, relative to the corresponding Ctrl mice. Note that the expression of *Foxj1* is also shown in Figure 33C (same dataset).

5.4.3.2 *Twnk* knockout affects the morphology of cilia in astrocytes

To further investigate ciliary phenotype upon Twinkle loss in astrocytes, we analysed morphology of cilia upon *Twnk* knockout in the brain and in cultured cells. To capture the maximum manifestation of the phenotype, we analysed mice at the preterminal stage of the phenotype development (4.5-5 months). To visualise cilia, we used immunostaining against the axonemal protein ARL13B. GFAP-positive astrocytes in the brain of Ctrl and TwKO^{astro} mice were monociliated (Figure 35A). To analyse cilia in astrocytes of both Ctrl and TwKO^{astro} brain, we used a costaining against ARL13B and ALDH1L1, and quantified the morphology of cilia in

ALDH1L1-positive cells. Cilia in the brain of Ctrl mice had variable morphology, which we classified as straight, bent, and contorted (Figure 35B). We found cilia of the same classes in TwKO^{astro} brain, of which the proportion of cilia with contorted morphology was increased (Figure 35C). Contorted cilia in TwKO^{astro} mice included corkscrew-like and S-shaped (Figure 35B). We also found very long cilia with several loops (Figure 35B, lowest panel). Such extreme morphologies were rare in TwKO^{astro} mice, and never occurred in Ctrl mice. Maximum and minimum length of cilia were 2.0 and 6.5 µm in Ctrl brain; 2.5 and 8.2 µm in TwKO^{astro} mice; and there was a shift in distribution towards longer cilia in astrocytes of TwKO^{astro} mice compared to Ctrl (Figure 35D).

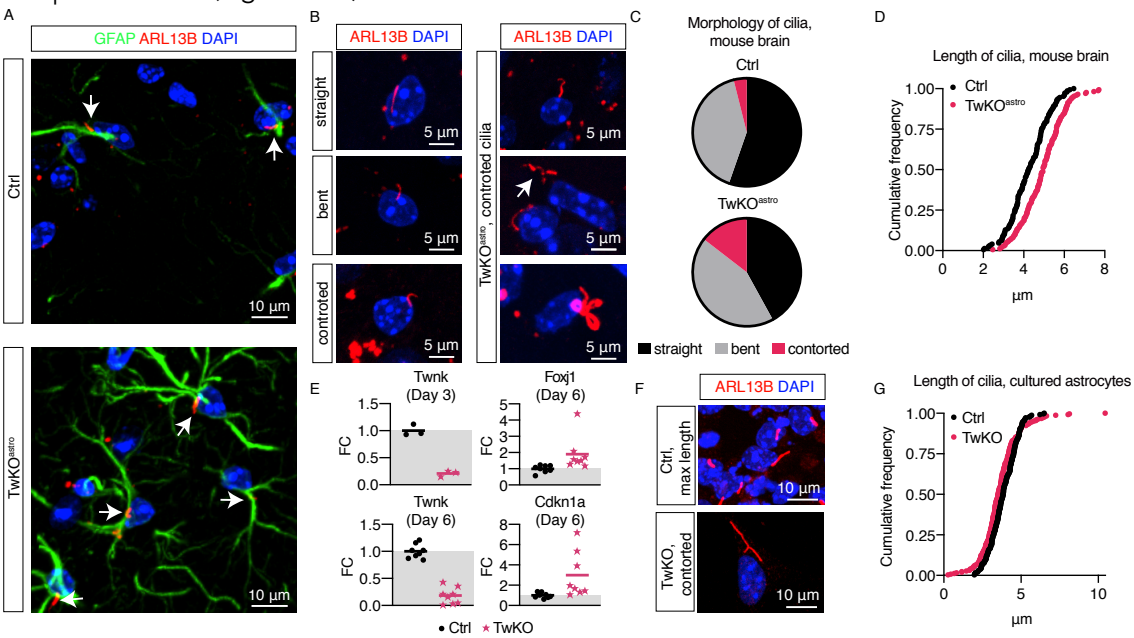

Figure 35. Morphology of cilia in TwKO^{astro} mouse brain and TwKO cultured astrocytes compared to Ctrl (from (III) and unpublished data). **(A):** Immunostaining against ARL13B and GFAP, mouse brain cortex, 4.5-5 months old mice. **(B):** Examples of the morphology of cilia in the mouse brain, cilia of ALDH1L1-positive cells are shown. **(C-D):** Quantification of the length and morphology of cilia in astrocytes (ALDH1L1-positive cells) in TwKO^{astro} and Ctrl brain cortex. n=5 mice per genotype; total number of cilia analysed in Ctrl = 148, in TwKO^{astro} = 174. Kolmogorov-Smirnov test: p-value < 0.0001, D = 0.5703. **(E):** Cultured astrocytes, RT-qPCR, symbols indicated replicates pooled from several independent experiments. Indicated days are days after viral transduction. TwKO = *Twnk* knockout. **(F):** Cultured astrocytes, immunostaining against ARL13B. Maximum length of cilia in Ctrl and a contorted cilium in TwKO cultures are shown. **(G):** Quantification of the length of cilia in cultured astrocytes. Data are pooled from two replicates per condition, 250 Ctrl and 250 TwKO cilia in total were analysed. Kolmogorov-Smirnov test: p-value = 0.0046, D = 0.1560. (A, B, E): MIP, confocal images. FC is a fold change ratio, relative to the corresponding controls. Imaging and quantification of cilia morphology in the brain by Satu Malinen.

To test whether ciliary response was dependent on the cell niche, we induced *Twnk* knockout in cultured astrocytes. To preserve physiological non-reactive astrocyte phenotype and low rate of cell division, we cultured cells in the chemically defined media without added serum (see 4.4). The majority of cultured astrocytes were monociliated, making this model

suitable for our study (data not shown). *Twnk* expression was downregulated already at three days after transduction of *Twnk*$^{loxp/loxp}$ cultures with the Cre-expressing virus (Figure 35E). Next, we analysed the expression of *Foxj1* and *Cdkn1a*, expression of which was upregulated in astrocytes purified from TwKO^{astro} mice (Figure 34D). In cultured astrocytes, we observed upregulated expression of these genes, but it was variable (Figure 35E). We observed some cilia with very contorted morphology in TwKO but not in Ctrl cultures (Figure 35F), which is similar to our observations in the brain (Figure 35B). Similar to changes in the morphology of cilia in TwKO^{astro} brain, the maximum length of cilia in TwKO astrocytes was increased compared to Ctrl (maximum length of cilia was 6.5 μm in Ctrl culture and 10.4 μm in TwKO culture) (Figure 35G). Additionally, some cilia in TwKO cultures were much shorter compared to Ctrl (minimum length of cilia was 2 μm in Ctrl culture and 0.28 μm in TwKO culture) (Figure 35G). It however remains to be established if this signal comes from shorter or aberrant cilia, or if these were protein aggregates. Collectively, this indicates that remodelling of a ciliary program is inherent to astrocytes.

5.4.3.3 Ciliation is preserved upon *Twnk* knockout in multiciliated ependymal cells

Ciliary remodelling in monociliated cortical astrocytes prompted us to investigate whether multiciliated ependymal cells were also affected by *Twnk* knockout. GFAP-73.12 promoter, which drives Cre expression in cortical astrocytes of TwKO^{astro}, was active in the ependymal cells (Figure 36). The ependymal cell layer was intact at the preterminal stage of the phenotype development, and ciliation was preserved (Figure 36). We also imaged ependymal cells using scanning electron microscopy and found that ciliation in TwKO^{astro} mice was comparable with Ctrl mice (Figure 36). In summary, these data show that fitness of ependymal cells is not affected by *Twnk* knockout, and the ciliation is preserved.

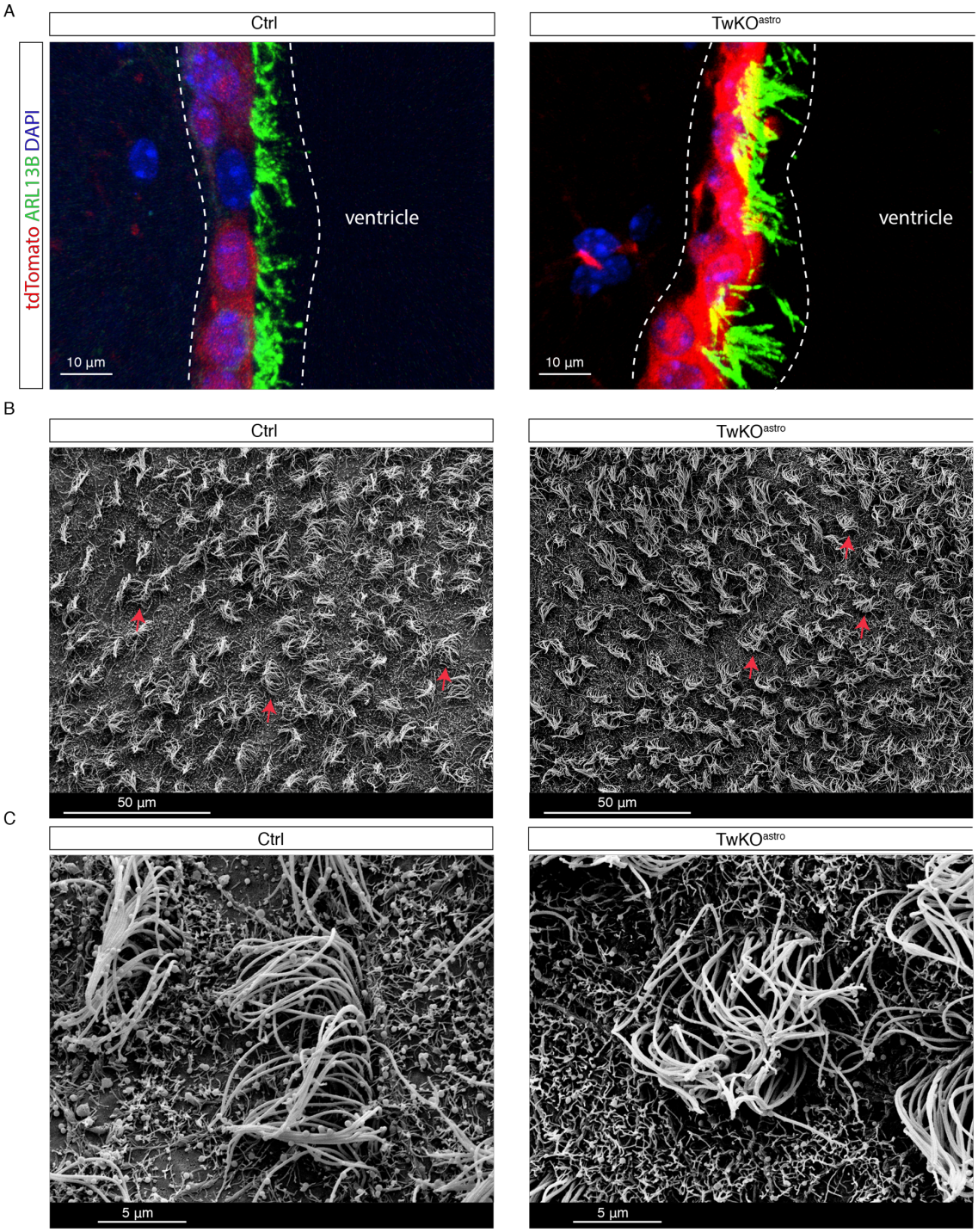

Figure 36. Ciliation of ependymal cells in TwKO^{astro} mice at the preterminal stage (4.5-5 months). **(A):** reporter mice, tdTomato is expressed upon Cre activity (see 4.2). Immunostaining against ARL13B. MIP, confocal images. **(B-C):** scanning electron microscopy photographs. Red arrows show tufts of cilia.

5.5 Motile ciliogenesis program is regulated in astrocytes upon various stimuli

Next, we asked whether the motile ciliogenesis program is regulated in reactive astrocytes induced by other stimuli and in other mouse models with mitochondrial dysfunction. We analyzed the expression of motile cilia, ciliogenic and multiciliated cell differentiation factors in published datasets (Figure 37). Expression of 61 out of 89 genes was upregulated in astrocytes purified from TwKO^{astro} mice and one gene was downregulated (Figure 37). Cultured astrocytes starved from HBEGF trophic factor or upon inhibition of EGFR signalling (Jiwen Li et al. 2019), displayed a robust upregulation of the motile ciliogenesis program, including induction of *Foxj1* (Figure 37). In turn, astrocytes stimulated with the pro-inflammatory IL-1**α**+TNF+C1q cocktail ((Liddelow et al. 2017), see 2.2.1.2), as well as reactive astrocytes sorted from mice with spinal cord injury (Anderson et al. 2016), displayed partial downregulation of the transcriptional program (Figure 37). There were no changes in astrocytes sorted from the aged mouse brain (Figure 37) (Boisvert et al. 2018). Knockout of *Twnk* or other genes involved in mitochondrial genome replication and expression in the heart (Kühl et al. 2017) did not induce this ciliogenic program, signifying cell specificity of the response (Figure 37). Together, our data show that motile ciliogenesis is a program regulated under various stress conditions in astrocytes.

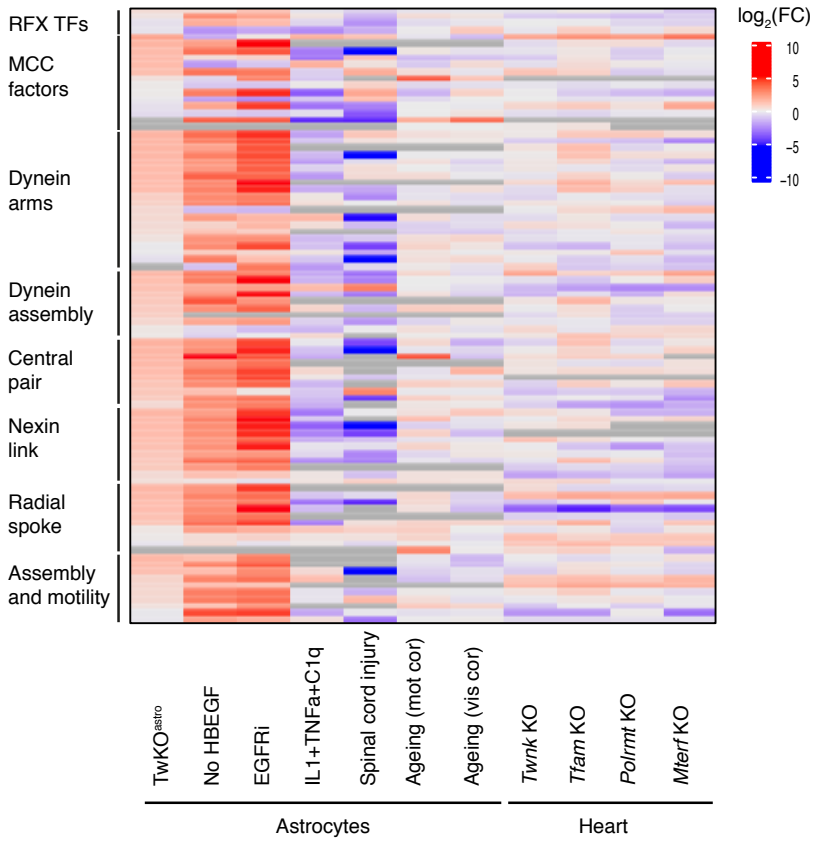

Figure 37. Regulation of the motile ciliogenesis program in astrocytes upon various insults and upon tissue-specific mitochondrial dysfunction. MCC = multiciliated cell. FC = fold change. Datasets are from this study (see Figure 28) and (Anderson et al. 2016; Liddelow et al. 2017; Jiwen Li et al. 2019; Boisvert et al. 2018; Kühl et al. 2017).

6 DISCUSSION

6.1 Cell-specific contribution to the pathogenesis of mitochondrial dysfunction in the CNS (I-III, unpublished data)

To investigate the pathogenesis of mitochondrial dysfunction in the CNS, we induced cell-specific conditional knockout of mitochondrial factors in mouse brain astrocytes and neurons (I-III). In this chapter, I discuss our findings in the light of literature which used similar approaches to investigate consequences of cell-specific mitochondrial dysfunction in the CNS.

When this thesis was initiated, it was already well established that neuronal homeostasis is dependent on mitochondrial function (Sörensen et al. 2001; Diaz et al. 2012; Fukui and Moraes 2009; Pickrell et al. 2011). In our experiment, we also observed that mitochondrial dysfunction in mouse brain neurons led to cell degeneration (see 5.1.3). At the time, there was no published research on consequences of mitochondrial dysfunction in astrocytes in the mammalian brain, however evidence from cultured cells suggested that astrocytes are less vulnerable to OXPHOS dysfunction than neurons (Pauwels, Opperdoes, and Trouet 1985; Almeida et al. 2001). We found that mitochondrial dysfunction in mouse brain astrocytes is sufficient to drive reactive astrogliosis and leads to a distinct pathology (see 5.1.4-5.1.6). Specifically, conditional knockout of *Twnk* or *Cox10* in astrocytes resulted in spongiotic pathology, whereas knockout of *Twnk* in neurons did not result in such pathology. Consequently, we propose that astrocytes can be primary contributors to the pathogenesis of human mitochondrial encephalopathies. The schematic of phenotypes observed upon knockout of *Twnk* in mouse brain astrocytes (TwKO^{astro}) and neurons (TwKO^{neuro}) is summarised in Figure 38.

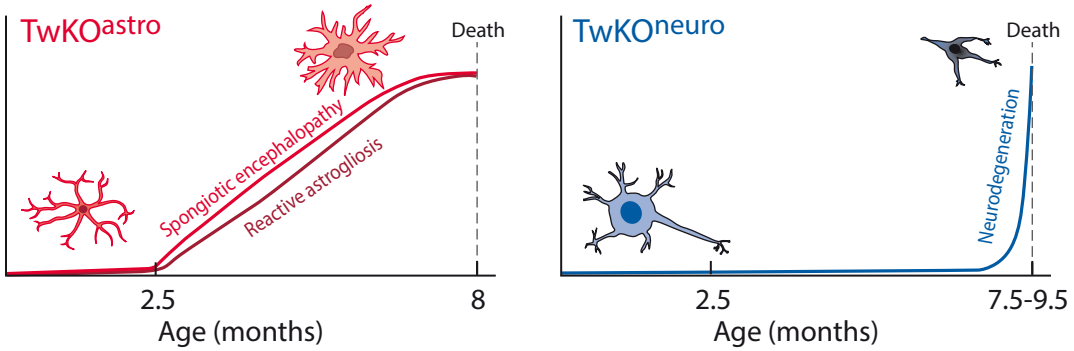

Figure 38. Schematic summary of TwKO^{astro} and TwKO^{neuro} phenotypes (modified from (I)).

6.1.1 Neurons

Neurodegeneration is commonly observed in mitochondrial encephalopathies, prompting the view that pathogenesis is driven by mitochondrial dysfunction in neurons. This hypothesis was tested by inducing genetic knockouts of mitochondrial factors in mouse brain neurons. Several of these studies, as well as the research presented in this thesis, found that disruption of the mitochondrial gene expression or the function of OXPHOS results in neurodegeneration and is sufficient to cause mouse death (see 5.1.1-5.1.3, (Sörensen et al. 2001; Diaz et al. 2012; Pickrell et al. 2011; Fukui and Moraes 2009; Bolea et al. 2019)).

Disruption of mitochondrial gene expression or OXPHOS function in neurons. The phenotype of TwKO^{neuro} mice resembles that of mice with neuronal knockout of mtDNA transcription and packaging factor *Tfam* (see 5.1.1-5.1.3, (Sörensen et al. 2001)). In both models, loss of mitochondrial gene expression occurred weeks to months before neurodegeneration or motor symptoms. Mice suddenly manifested with severe distress resulting in mouse death several days after, and this coincided with *en mass* neurodegeneration (see 5.1.1-5.1.3, (Sörensen et al. 2001)). Consequently, it can be concluded that the primary consequences of Twinkle and TFAM loss in neurons are similar. Neuronal depletion of the Complex III catalytic subunit also resulted in acute neurodegeneration, which was followed by sudden mouse death at the age of 3-3.5 months (Diaz et al. 2012). Neuronal *Cox10* knockout led to OXPHOS deficiency by two months of age, manifesting with progressive neurodegeneration from four months of age until mouse death at 10-12 months (Diaz et al. 2012). All these studies, including ours, used CamKII**α**-Cre promoters, which target excitatory neurons of the CA1 hippocampal area and cortex ((Xu et al. 2000; Tsien et al. 1996), see 4.2). It is possible that mitochondrial dysfunction in other neuronal subtypes would manifest differently. An important consideration in the interpretation of these findings is also that CamKII**α**-Cre can be expressed in peripheral tissues (see 2.4, 4.1). Mitochondrial dysfunction in peripheral tissues, including possible cell death, may contribute to morbidity phenotypes.

Twnk vs Tfam knockout in neurons. Mice with neuronal *Tfam* knockout manifested 1.5-4 months earlier than *Twnk* knockout, although both studies used CamKII**α**-Cre drivers (see 5.1.1-5.1.3, (Sörensen et al. 2001; Dufour et al. 2008)). Published evidence suggests that mitochondrial gene expression loss in neurons manifests in a quantitative manner. Firstly, the severity of phenotypes in mosaic chimeras with neuronal *Tfam* depletion was dependent on the level of genetic recombination (Dufour et al. 2008). Secondly, the severity of motor phenotypes caused by nuclease-induced cleavage of mtDNA in neurons was dependent on the level of mitochondria-targeted endonuclease expression (Pickrell et al. 2011; Fukui and Moraes 2009). The timeline of manifestations in *Twnk* compared to *Tfam* knockout might depend on the dynamics of Twinkle and TFAM protein loss, as well as the mitochondrial genome expression loss. To date, these were not investigated in parallel. An important difference between the models is that the loss of TFAM can both directly affect the stability of mtDNA and impede mitochondrial transcription (reviewed in (D. Kang, Kim, and Hamasaki 2007; Gustafsson, Falkenberg, and Larsson 2016)), while loss of Twinkle leads to failures in mitochondrial genome replication (Milenkovic et al. 2013). In cardiac tissue, knockout of *Tfam* in the heart also manifested earlier and led to shorter lifespan than analogous *Twnk* (Kühl et al. 2017). In principle, cell responses to Twinkle and TFAM depletion also may diverge, contributing to the disease course. Conditional knockout of these proteins in cardiac tissue however resulted in largely overlapping gene expression responses (Kühl et al. 2017).

Together, this body of research provided important evidence that neurons can cope with mitochondrial dysfunction for prolonged periods of time, but eventually succumb to it. Investigating the mechanisms that underlie this switch might provide new strategies to combat neurodegeneration caused by mitochondrial dysfunction.

6.1.2 Astrocytes

An increased level of GFAP expression is a common finding in mitochondrial encephalopathies. In mouse models with knockout of mitochondrial proteins in neurons,

astrocytes displayed increased GFAP expression (referred to as reactive astrogliosis) (Sörensen et al. 2001; Diaz et al. 2012; Pickrell et al. 2011; Fukui and Moraes 2009; Bolea et al. 2019). These findings supported the hypothesis that reactive astrogliosis in mitochondrial encephalopathies may constitute a cell nonautonomous response. However, we found that astrocytic loss of Twinkle (TwKO^{astro}) or COX10 (Cox10KO^{astro}) drove reactive astrogliosis (see 5.1.4-5.1.6). Additionally, both TwKO^{astro} and Cox10KO^{astro} mice manifested with spongiotic encephalopathy, while TwKO^{neuro} did not. Together, this highlights the self-governing contribution of astrocytes to the pathogenesis of mitochondrial dysfunction in the CNS.

Disruption of mitochondrial function in astrocytes. In recent years, several other studies investigated consequences of conditional knockout of mitochondrial factors in mouse brain astrocytes (Table 2). Of these, knockout of *Tfam*, as well as *Afg3l1 and Afg3l2* genes encoding protein quality control m-AAA protease, resulted in increased GFAP expression in astrocytes (Table 2, (Fiebig et al. 2019; Murru et al. 2019)). The loss of either Twinkle or TFAM ultimately results in the loss of the mitochondrial gene expression (see 6.1.1). Loss of m-AAA protease function impedes mitochondrial proteostasis and leads to the loss of mitochondrial ribosomes (Almajan et al. 2012; Richter et al. 2019). Morbidity and lifespan differed in these models, and the loss of *Afg3l1* and *Afg3l2* was the most severe (Table 2). Importantly, only the loss of *Afg3l1* and *Afg3l2* resulted in degeneration of a proportion of targeted astrocytes, whereas astrocytes survived through depletion of other mitochondrial proteins (Table 2). Similar to neuronal Cre drivers, astrocytic Cre drivers are also expressed in peripheral tissues, which possibly contributes to the observed morbidity phenotypes (see 2.4, 4.1).

Table 2. *Mouse models with conditional knockout of mitochondrial factors in astrocytes. HEP = humane endpoint. P4-P35 = postnatal day at which Cre expression is induced.*

Gene	Phenotype	Cre induction	Promoter	Reference
Twnk (mtDNA helicase)	Reactive astrogliosis (multiple markers ↑) Reactive microgliosis (IBA1) Spongiotic encephalopathy Mitochondrial swelling Weight loss (HEP at 5-6 months)	P4	GFAP73.12 (Jax012886)	(Ignatenko et al. 2018)
Cox10 (Complex IV assembly factor)	Reactive astrogliosis (GFAP↑) Spongiotic encephalopathy Weight loss (HEP at 4 months) or stalled weight gain	P4	GFAP73.12 (Jax012886)	This thesis, unpublished
Cox10 (Complex IV assembly factor)	No reactive gliosis No signs of pathology Increased glycolysis	P30-P35	*GLAST*^{CreERT2} (MGI:3830051)	(Supplie et al. 2017)
Tfam (mtDNA transcription and packaging factor)	Reactive astrogliosis (GFAP↑) Mitochondrial swelling	P14-18	*GLAST*^{CreERT2} (MGI:3830051)	(Beckervordersandforth et al. 2017; Fiebig et al. 2019)
Afg3l2 (*m*-AAA protease subunit)	Reactive astrogliosis (GFAP↑) Bergmann glia dislocation Mitochondrial swelling Reduced body weight	P7-11	hGFAP^{ERT} (Chow et al., 2008)	(Murru et al. 2019)
Afg3l2 and *Afg3l1* (*m*-AAA protease)	Reactive astrogliosis (GFAP↑) Reactive microgliosis (IBA1) Bergmann glia dislocation and degeneration Mitochondrial swelling Stalled body weight gain Motor dysfunction HEP at 4 weeks	P7-11	*Afg3l2:* hGFAP^{ERT} (Chow et al., 2008) *Afg3l1:* constitutive (S. Wang et al. 2016)	(Murru et al. 2019)
Ucp2 (mitochondrial uncoupling protein)	Increased mitochondrial area Pathology is not investigated	6 weeks	hGFAP^{ERT2} (Jax012849)	(García-Cáceres et al. 2016)
Mfn2 (mitochondrial fusion factor)	No reactive gliosis Fragmented mitochondrial network Reduced mitochondrial density	Adult	hGFAP^{ERT} (Chow et al., 2008)	(Göbel et al. 2020)
Mfn1 (mitochondrial fusion factor)	No apparent phenotype Pathology is not investigated	Adult	hGFAP^{ERT} (Chow et al., 2008)	(Göbel et al. 2020)

Variability of phenotypes caused by Cox10 loss. Our findings using Cox10KO^{astro} mice contradict with findings using another mouse model of astrocytic C*ox10* knockout (see 5.1.6, Table 2, (Supplie et al. 2017)). This published model did not manifest with any evident brain pathology, reactive astrogliosis, or morbidity (Table 2, (Supplie et al. 2017)). Our Cox10KO^{astro} mice displayed reactive astrogliosis, spongiotic encephalopathy, and weight loss (see 5.1.6). Several factors can account for the difference between phenotypes:

a) Genetic background of inbred mouse strains. The C57BL/6 substrain used in (Supplie et al. 2017) is not specified; Cox10KO^{astro} and TwKO^{astro} manifest similarly and are inbred C57BL/6JOlaHsd strains (see 4.1, 5.1.4-5.1.6). In addition to the inbred background, TwKO^{astro} mice were maintained also on mixed genetic backgrounds, originating from reporter mice and from the recently rederived *Twnk*$^{loxp/loxp}$ mice (see 4.1, JAX: 007576). Irrespective of genetic background, mice always manifested with an increased GFAP expression, spongiotic encephalopathy, and progressive body weight loss (data not shown). This rules out with high confidence the contribution of C57BL/6JOlaHsd background to the observed phenotypes.

b) Subpopulations of astrocytes targeted by Cre expression. In Cox10KO^{astro} mice, Cre is driven by GFAP73.12-Cre (see 4.2), while the study (Supplie et al. 2017) used GLAST^{CreERT2} driver. These drivers could induce *Cox10* knockout in distinct, non-overlapping astrocyte subpopulations, which would manifest differently. Both drivers are expressed in cerebellar Bergmann glia and in cerebellar astrocytes, however only Cox10KO^{astro} mice presented in this thesis manifested with spongiotic pathology and increased GFAP expression (see 5.1.6, (Supplie et al. 2017)). Increased GFAP expression and spongiotic pathology were observed in the cortex, hippocampus, and cerebellum of Cox10KO^{astro} and TwKO^{astro} mice, and in both gray and white matter (data not shown). Considering the overlapping patterns of GFAP73.12-Cre and GLAST^{CreERT2} expression, as well as the uniformity of Cox10KO^{astro} and TwKO^{astro} manifestation across Cre-expressing astrocytes, it is unlikely that the difference between mouse models is explained by Cre expression in distinct astrocyte subpopulations.

c) Developmental stage of Cre induction. A critical difference between these models is timing of Cre induction. GFAP73.12-Cre starts to express spontaneously at postnatal day four (Table 2). This age corresponds to the astrogenesis stage, when cells divide and migrate (Clavreul et al. 2019), see 2.1.1). In GLAST^{CreERT2}, Cre expression is induced by tamoxifen administration at postnatal day 30. At this age, astrogenesis is completed ((Clavreul et al. 2019), see 2.1.1). This introduces an intriguing possibility that during early postnatal development mitochondrial dysfunction in astrocytes triggers progressive pathology, while mature astrocytes can cope with a similar insult. Testing of this hypothesis would require investigating the consequences of COX10 loss at different developmental stages, which could be achieved by earlier tamoxifen administration to *Cox10*$^{loxp/loxp}$ GLAST^{CreERT2} mice.

Finally, t is also theoretically possible that tamoxifen administration was protective in *Cox10* mice generated using GLAST^{CreERT2} promoter, or that different patterns of GFAP73.12-Cre and GLAST^{CreERT2} expression in peripheral tissues contributed to the differences in phenotypes.

Mitochondrial dysfunction in mature vs neonatal astrocytes. As discussed above, neonatal knockout of *Twnk* or *Cox10* manifests with spongiotic encephalopathy and an increased GFAP expression, whereas knockout of *Cox10* in mature astrocytes is asymptomatic (see 5.1.6, Table 2, (Supplie et al. 2017)). Conditional knockout of mitochondrial fusion factors *Mfn1* and *Mfn2*, or *Ucp2* gene, which encodes mitochondrial uncoupling protein in mature astrocytes was not associated with brain pathology, morbidity or reactive astrogliosis (Table 2, (García-Cáceres et al. 2016; Göbel et al. 2020)). This further highlights the ability of mature astrocytes to cope with mitochondrial dysfunction. Knockout of Complex I or Complex II subunits in neural stem cells during mouse embryonic development resulted in a defect of neuronal, but not astrocytic, differentiation (Díaz-Castro et al. 2015; Quintana et al. 2010; Cabello-Rivera et al. 2019). These OXPHOS-deficient astrocytes displayed an increased expression of GFAP, which only occurred postnatally (Díaz-Castro et al. 2015; Quintana et al. 2010). These data, together with our findings, are consistent with the interpretation that astrocytes with mitochondrial dysfunction can survive, but respond with reactive astrogliosis; and if mitochondrial dysfunction occurs during developmental stages, it can result in brain pathology. The only exception to astrocyte fitness upon mitochondrial dysfunction is the early postnatal loss of *Afg3l1* and *Afg3l2*, as mice displayed a modest cell degeneration (Murru et al. 2019).

Mechanistically, loss of mitochondrial function resulting from a gene knockout can occur sooner in dividing cells due to more rapid depletion of protein amount. Dividing cells also have higher biosynthetic demands, as mitochondrial biogenesis is required for cell division, and loss of mitochondrial genome expression often induces a cell cycle arrest (reviewed in (Arakaki et al. 2006; Antico Arciuch et al. 2012; Battersby and Richter 2013)). Finally, transcriptional responses to mitochondrial dysfunction differ in dividing cells compared to quiescent (Mick et al. 2020). The induction of such responses may occur and contribute to pathogenesis even before the detectable loss of OXPHOS function.

Taken together, it is plausible to assume that the stage of astrogenesis at which mitochondrial dysfunction occurs plays a crucial role in phenotypic manifestation. Testing this hypothesis promises to refine both mitochondrial roles in astrocyte homeostasis and the mechanisms behind manifestations of mitochondrial encephalopathies with astrocytic involvement.

6.1.3 Ependymal cells

Using TwKO^{astro} mice, we investigated the consequences of *Twnk* loss in ependymal cells. Cells coped with mitochondrial dysfunction, as the ependymal cell layer was intact even at the preterminal stage of the phenotype progression, and ciliation was preserved (see 5.4.3.3).

Coordinated beating of ependymal cilia is ensured by multiprotein axonemal dynein motors with ATPase properties (reviewed in (King 2016)), and is dependent on the ATP supply (Chen et al. 2015; Boulais et al. 2015). Disruption of ciliary beating manifests as hydrocephalus in mice (Lechtreck et al. 2008; Ibañez-Tallon et al. 2004; Tissir et al. 2010; Appelbe et al. 2013; Muniz-Talavera and Schmidt 2017). We did not investigate the motility of ependymal cilia in TwKO^{astro} mice, however absence of hydrocephalus would be consistent with the preservation of ependymal cilia function. To date, I was not able to locate other published studies investigating how mitochondrial dysfunction affects ependymal cells.

6.1.4 Oligodendrocyte lineage cells

Findings in patients with mitochondrial encephalopathies include myelin abnormalities, suggesting that oligodendrocyte function is affected (reviewed in (Bindu et al. 2018)). Experiments using genetically modified mice to disrupt mitochondrial function in oligodendrocyte lineage cells revealed that manifestations are dependent on both the developmental stage and the specific molecular defect. Knockout of *Cox10* in mature oligodendrocytes was asymptomatic (Fünfschilling et al. 2012). In turn, loss of m-AAA protease (*Afg3l1* and *Afg3l2* knockout) in mature oligodendrocytes resulted in cell death, demyelination, mitochondrial swelling, axonal degeneration, reactive astrogliosis (assessed using immunostaining against GFAP), and microgliosis (assessed using immunostaining against IBA1), motor phenotype, and reduced weight gain (S. Wang et al. 2016). Knockout of *Cox10* in dividing precursors of both brain oligodendrocytes and peripheral Schwann cells did not result in cell death or reactive astrogliosis (assessed using immunostaining against GFAP), however it led to peripheral hypomyelination, neuropathy and motor deficiency (Fünfschilling et al. 2012). Collectively, this research has established that cells of oligodendrocyte lineage have self-governing contribution to the pathogenesis of mitochondrial dysfunction in the nervous tissue.

6.1.5 Microglia

Brain pathology in mitochondrial diseases often presents with reactive microgliosis (reviewed in (Lake et al. 2015)). Similar to astrogliosis, this is commonly interpreted as a cell nonautonomous response to the pathology. Mitochondrial dysfunction in astrocytes or neurons also leads reactive microgliosis in the mouse brain ((I), (Murru et al. 2019)).

Studies investigating the consequences of mitochondrial dysfunction in microglia seem absent in published literature. It is however known that inflammatory responses of immune cells (including microglia) are closely associated with massive metabolic reprogramming (reviewed in (Harry et al. 2020; Gordon et al. 2008)). There is overwhelming evidence for the metabolic regulation of immune cell functions, and the activity of the field is reflected by the emergence in recent years of the term immunometabolism (reviewed in (Paolicelli and Angiari 2019)). Thus, it is plausible to assume that mitochondrial dysfunction can affect microglial homeostasis in health and disease. The experiments depleting microglial cells in various mouse models have demonstrated that these cells can have both protective and harmful roles in pathology, however no such experiments were done in a setting of mitochondrial dysfunction (Green, Crapser, and Hohsfield 2020)). To conclude, microglial contribution to the pathogenesis of mitochondrial dysfunction is, perhaps surprisingly, completely unknown.

6.2 Spongiotic encephalopathy (I, II, unpublished data)

6.2.1 Cellular basis of the pathology

TwKO^{astro} and Cox10KO^{astro} mice manifested with brain pathology, which throughout this thesis is referred to as spongiotic encephalopathy (see 5.1.5-5.1.6). Disruption of mitochondrial function in other than astrocytes cell types did not cause spongiotic encephalopathy in the mouse brain (see 6.1). In this chapter, I discuss these findings in the light of similar pathologies observed in human diseases and other mouse models, focusing on phenotypes caused by mitochondrial dysfunction.

Examination of paraffin brain sections of TwKO^{astro} and Cox10KO^{astro} mice stained with hematoxylin and eosin revealed progressive vacuolation of brain parenchyma (see 5.1.5-5.1.6). The regional manifestation in TwKO^{astro} and Cox10KO^{astro} mice was different, despite the fact that mice were generated using the same Cre driver: the spongiotic pathology in the cortex was similar, while in the cerebellum it was more severe in Cox10KO^{astro}. This suggests higher susceptibility of cerebellar astrocytes and Bergman glia to COX10 loss compared to cortical astrocytes. This may evidence also higher susceptibility of cerebellar astrocytes and Bergman glia to COX10 compared to Twinkle loss, however, rates of the protein and the OXPHOS function loss may be different in these models but were not investigated in parallel.

The importance of ultrastructural investigation of spongiotic encephalopathies. At the cellular level, light microscopy analysis of TwKO^{astro} mice revealed vacuoles of oval and round shapes (see 5.1.5-5.1.6). These were ~1 to 15 µm in diameter and appeared empty or contained filament-like structures. The pathology was progressive from scarce vacuoles at the age of 2-3 months to mesh-like areas by 7-8 months. At the ultrastructural level, transmission electron microscopy revealed several types of electron-light areas: i) round and oval shapes up to 15 µm in diameter, which appeared empty or contained debris; ii) swollen mitochondria up to 4 µm in diameter containing electron-light areas; iii) vacuolated neuronal axons (see 5.1.2, 5.1.5). These abnormalities may contribute to the vacuolation of brain parenchyma observed with light microscopy. A detailed investigation in younger mice using volumetric electron microscopy can provide a better understanding of these abnormalities. Cell death is a simple explanation to account for the empty space, however, we often observed vacuoles in cells with an intact nucleus. Furthermore, we did not observe a decrease in astrocyte number (see 5.1.4), and in animal models with astrocytic cell death vacuolation was not reported (Cui et al. 2001; Schreiner et al. 2015). Vacuoles were not stained with bodipy lipid staining or in any immunostainings, however, GFAP-positive filaments often surrounded these vacuoles (see 5.1.5). Generally, such pathology can be compatible with disruptions in osmotic balance at the cellular level, possibly arising first in mitochondria.

Similar pathologies were observed with light microscopy upon knockout of genes encoding gap junction proteins in astrocytes (discussed in 2.1.5.1). Examination of the pathology using electron microscopy revealed the presence of edematous astrocytes, as well as vacuolation within oligodendrocytic cytoplasm and of myelinated axons (Lutz et al. 2009). These vacuoles often had an irregular shape, and there was no swelling of mitochondria in astrocytes (Lutz et al. 2009). These findings differ from TwKO^{astro} mice, suggesting that the cellular basis of the pathology, despite the similarity of light microscopy findings. Thus, it can be concluded that at the level of light microscopy unrelated vacuolation pathologies can appear similarly. Thus, the electron microscopy provides a better insight to understand the cellular basis of spongiotic encephalopathies and other vacuolation pathologies.

Mitochondrial spongiotic encephalopathies. At the level of light microscopy, similar to TwKO^{astro} mice pathology occurs in several mitochondrial human diseases (Nolte et al. 2013; Sofou et al. 2012). Such pathologies have been described as 'spongiotic encephalopathy', 'spongiosis', 'spongiform encephalopathy', 'vacuolation', 'vacuolization', and 'cavitation'. The cellular or organellar basis of these pathologies remains mostly unknown. The electron microscopy examinations of human spongiotic encephalopathies are however rare, and are only possible postmortem, when profound damage to the tissue has occurred (for example, (Sandbank and Lerman 1972; Vuia 1975)). It is therefore challenging to definitively conclude whether spongiotic pathology in human mitochondrial encephalopathies has a similar cellular basis as in TwKO^{astro} and Cox10KO^{astro} mice.

Evidence that pathology of TwKO^{astro} and Cox10KO^{astro} mice arises by the same mechanism as in human mitochondrial encephalopathies could emerge from human genetics. To date, I was unable to locate reports where pathogenic variants in *TWNK* or *COX10* result in human spongiotic encephalopathies (literature and OMIM database search). It however should be noted that mitochondrial diseases are rare genetic diseases, and the published spectrum of pathologies arising from pathogenic variants in a given gene may be incomplete. Out of the mtDNA replication machinery components (see 2.3.4), pathogenic variants in *POLG* can result in spongiotic encephalopathy in the brain ((Palin et al. 2012; Palin, Paetau, and Suomalainen 2013), reviewed in (Sofou et al. 2012; Harding 1990)) and in the spinal cord (J. Nikkanen, Landoni, and Balboa 2018). Pathogenic variants in genes encoding subunits of OXPHOS complexes (including Complex IV) can cause Leigh syndrome, which also is associated with spongiotic encephalopathy in a number of cases (reviewed in (Lake et al. 2015; Carlo Viscomi, Ardissone, and Zeviani 2016), discussed in 2.3.6.1). A comprehensive investigation of the genetic background of human spongiotic encephalopathies would help to establish an array of molecular events which can be causative of this pathology. To study astrocyte contribution to spongiotic encephalopathies, an approach to induce in mice cell-specific expression of a pathogenic variant known to cause human spongiotic encephalopathy could be used.

Spongiotic encephalopathy in mouse models of OXPHOS dysfunction. In addition to TwKO^{astro} and Cox10KO^{astro} mice, spongiotic pathology was reported for the following mouse models with disrupted OXPHOS function: i) *Ndufs4* knockout, both constitutive and specific to radial glial cells ((Quintana et al. 2010); model of Leigh syndrome, see 2.3.6.2), ii) *Coq9* knockin with a patient pathogenic variant ((García-Corzo et al. 2013), see 2.3.6.2). Coenzyme Q is the part of the respiratory chain (see 2.3.2). Thus, an impaired OXPHOS function could be the unifying link between *Coq9*, *Twnk, Cox10*, and *Ndufs4* loss.

Findings in TwKO^{astro} mice suggest coenzyme Q biosynthesis deficiency, including decreased expression of *Coq9* but not of other *Coq* genes (see 5.4.2.2). It is of interest that *Coq9* mutant mice with primary coenzyme Q deficiency and TwKO^{astro} mice with secondary coenzyme Q biosynthesis deficiency manifest with spongiotic encephalopathy, coinciding with reactive astrogliosis and microgliosis. Deficiency of the coenzyme Q biosynthesis in TwKO^{astro} mice is consistent with findings in mice with cardiac knockout of *Twnk* and four other mitochondrial proteins, which revealed coenzyme Q deficiency as a major consequence of mitochondrial genome expression loss (Kühl et al. 2017). Taken together, it would be intriguing to investigate the contribution of the secondary coenzyme Q deficiency to the brain pathology that occurs in TwKO^{astro} mice. It should be noted that to date no detailed electron microscopy investigation was performed for Cox10KO^{astro} mice or published for *Coq9* and *Ndufs4* knockout mice, thus the cellular basis of these pathologies remains elusive.

6.2.2 Treatment

No effective therapeutics to combat human spongiotic encephalopathies exist. Several treatment strategies were effective to extend lifespan and attenuate reactive gliosis of *Ndufs4* knockout mice, however effects on tissue architecture and vacuolation were not investigated in these studies (see 2.3.6.4). Administration of a reduced form of coenzyme Q (ubiquinol-10) or its structural analogue **β**-resorcylic acid to *Coq9* mutant mice extended lifespan, modulated morbidity phenotypes, and markedly reduced signs of spongiotic encephalopathy and reactive astrogliosis (assessed using immunostaining against GFAP), but not reactive microgliosis (assessed using immunostaining against IBA1) (Hidalgo-Gutiérrez et al. 2019; García-Corzo et al. 2014). This demonstrates the effectiveness of metabolite supplementation for treatment of spongiotic pathologies resulting from coenzyme Q deficiency.

TwKO^{astro}, *Ndufs4* knockout, and *Coq9* mutant mice were administered with rapamycin (see 5.3, 2.3.6.4). In all three models, high dosage of rapamycin led to reduced body weight due to impaired growth or accelerated weight loss (see 5.3, (Johnson et al. 2015; Barriocanal-Casado et al. 2019)). Rapamycin administration extended lifespan and modulated morbidity of *Ndufs4* knockout mice, as well as attenuated reactive gliosis. The effects on vacuolation of brain parenchyma were however not reported. In contrast, rapamycin did not improve morbidity or affected spongiotic pathology in TwKO^{astro} and *Coq9* mutant mice (see 5.3, (Barriocanal-Casado et al. 2019)). GFAP expression was attenuated in the diencephalon but not in other brain regions of *Coq9* mutant mice, and not affected in TwKO^{astro} mice. The variability of the intervention outcomes highlights the importance of using disease-specific models, closely replicating both the cause and manifestations of human diseases (also discussed in 2.3.6.2).

Finally, administration of a ketogenic diet to TwKO^{astro} mice led to aggravated spongiotic pathology, increased GFAP expression, and accelerated body weight loss (see 5.3). The mechanism for how astrocytes of TwKO^{astro} mice compensate for OXPHOS deficiency was not investigated, but is likely to involve increased glycolytic flux. Increased glycolytic flux is a general response of cells to mitochondrial dysfunction, and was also shown to occur in astrocytes upon *Cox10* knockout (Supplie et al. 2017). It would be logical to assume that depletion of dietary carbohydrates can impede this coping mechanism, further compromising energy metabolism. This highlights that dietary interventions in metabolic diseases should be done with caution.

6.3 New insights into astrocytic responses to mitochondrial dysfunction (II, III, unpublished data)

Using TwKO^{astro}, TwKO^{neuro}, and Cox10KO^{astro} mice, we investigated known cell stress responses to cell-specific mitochondrial dysfunction in the CNS. We also investigated the whole-genome transcriptional response of astrocytes purified from TwKO^{astro} mice. We discovered downregulated lipid biosynthesis and an induced motile ciliogenesis program in astrocytes of TwKO^{astro} mice, which we further elucidated using additional experimentation. In this chapter, I discuss these cell responses in the light of their potential effects to pathogenesis of mitochondrial dysfunction in the CNS.

6.3.1 Integrated stress response

There is overwhelming evidence that the ISR is activated in response to mitochondrial dysfunction in various model organisms, cultured human cells, and in patients with mitochondrial diseases (see 2.3.6.3). A partially overlapping response upon chronic mitochondrial dysfunction is defined as ISR^{mt} (see 2.3.6.3). Analysis of the gene expression signature showed that these responses were induced in TwKO^{astro} and Cox10KO^{astro} mice, but not in TwKO^{neuro} mice (see 5.2). RNA sequencing analysis of astrocytes purified from TwKO^{astro} mice revealed that the response was induced autonomously in the astrocytes (other cell types were not investigated) (see 5.4.1).

The novelty of our findings lies in the cell type specificity of responses to mitochondrial dysfunction in the CNS. The evolutionary conserved ISR signalling was not activated in neurons in response to mitochondrial dysfunction (see 5.2). The induction might be hindered when tissue lysates are analysed instead of purified cells, however the response was evident in TwKO^{astro} and Cox10KO^{astro} cortical lysates (see 5.2). Out of the investigated genes that are characteristic of the induced response, only *Atf3* expression was upregulated in TwKO^{neuro}. While the induction of *Atf3* expression in TwKO^{neuro} mice might be independent of ISR/ISR^{mt}, it evokes interest in its potential regulatory role, which could be further elucidated by manipulating *Atf3* expression and analysing its binding sites in TwKO^{neuro} mice.

Investigations of the role of these responses in the pathogenesis of mitochondrial dysfunction are surprisingly limited. Knockout of *Fgf21* and inhibition of mTorc1 activity with rapamycin in Deletor mice attenuated expression of several core ISR^{mt} genes and affected metabolic signature ((Forsström et al. 2019, Khan et al. 2017), Deletor mice are introduced in 2.3.6.2). Rapamycin treatment also resulted in a decreased proportion of muscle fibers with complex-specific OXPHOS deficiency (Khan et al. 2017). This suggested that *Fgf21* and mTorc1 modulate part of the ISR^{mt} in the muscle upon chronic mitochondrial dysfunction.

Fgf21 is not expressed in the brain, and was not induced in TwKO^{astro}, Cox10KO^{astro}, and TwKO^{neuro} mice (see 5.2, (II)). mTorc1 activity did not appear induced in TwKO^{astro}, at least when assessed by pS6/S6 ratio (II). Of note, both pS6 and S6 levels were markedly reduced in TwKO^{astro} mice, possibly signifying attenuation of translation (II). This suggests that ISR/ISR^{mt} induction in the brain can be independent of both *Fgf21* and mTorc1 activity. To investigate the ISR contribution to the pathogenesis of mitochondrial dysfunction in the CNS, mouse models manifesting with the ISR induction, brain pathology, and morbidity phenotypes (such as TwKO^{astro} and Cox10KO^{astro}) can be useful.

6.3.2 Lipid metabolism

Mitochondrial dysfunction induced a transcriptional response to inhibit expression of lipid biosynthesis in astrocytes of TwKO^{astro} mice at the early stage of pathology development, 3-3.5 months (see 5.4.2). Brain lipid homeostasis was profoundly dysregulated, as levels of over a hundred lipids and lipid-like molecules were changed in TwKO^{astro} mice compared to Ctrl, which included depletion of several lipid classes. At 4-8 months of age, we observed accumulation of lipid droplets in the brain of TwKO^{astro} mice (earlier time points were not yet investigated for this phenotype). In turn, lipid composition of TwKO^{neuro} mice was globally unaffected, although levels of several metabolites were changed. Such difference between the brain lipid composition of TwKO^{astro} compared to TwKO^{neuro} mice might indicate both the disparate cell responses to *Twnk* knockout of astrocytes compared to neurons, as well as differential contribution of these cell types to brain lipid homeostasis.

Lipids constitute about 40% of gray matter, 50% of white matter, and 80% of myelin in the brain by dry mass (O'Brien and Sampson 1965). Unlike fat tissue, which has high lipid content due to the lipid storage, presence of lipid droplets in a healthy brain is minimal. Brain is semi-autonomous in lipid biosynthesis. Essential fatty acids, as well as abundant in the brain docosahexaenoic and arachidonic acids, are supplied from periphery; cholesterol, many fatty acids, lipoproteins, and other lipid molecules are synthesized by brain cells. Lipid metabolism differs between brain cell types. This is reflected by the functional specialisation, including production of myelin by oligodendrocytes and the exceptional demands of neurons, such as synaptic membrane maintenance and synaptogenesis. Brain cells types also demonstrate considerable differences in their lipid composition (Fitzner et al. 2020).

Lipid biosynthesis. Astrocytes are the important contributors to lipid biosynthesis and degradation in the brain. Enzymes of both lipid biosynthesis and **β**-oxidation pathways are enriched in astrocytes [1,2]. Astrocyte activities include synthesis and export of cholesterol (Mauch et al. 2001; Nieweg, Schaller, and Pfrieger 2009; Hirsch-Reinshagen et al. 2004), production of lipoprotein particles (LaDu et al. 1998; Mauch et al. 2001; Chen et al. 2013), and **β**-oxidation (Eraso-Pichot et al. 2018; Ioannou et al. 2019; Auestad et al. 1991). Mice with impaired cholesterol biosynthesis in embryonic astrocytes manifested with microcephaly and behavioral abnormalities (Camargo et al. 2012; Ferris et al. 2017; Camargo et al. 2017). Thus, these experiments constitute an important piece of evidence of the importance of astrocyte lipid biosynthesis function. A consideration is however that a small number of neurons and oligodendrocytes were also targeted, which can have a confounding effect (Bajenaru et al. 2002). Mice with disrupted in astrocytes cholesterol biosynthesis displayed an accumulation of some dietary lipids in the brain (Camargo et al. 2012). Administration of a high-fat diet modulated morbidity phenotypes and resulted in decreased mortality of these mice, as well as partially rescued impaired myelination and conduction velocity (Camargo et al. 2012, 2017). This provided evidence that dietary fats can modulate dysregulation of lipid homeostasis in the brain, presenting a promising intervention strategy.

Lipid droplets. Our finding that lipid composition is profoundly affected in TwKO^{astro} brain highlights the importance of astrocyte function for brain lipid homeostasis, elucidating the lipid classes most dependent on astrocyte function (see 5.4.2.1). Despite profound downregulation of lipid biosynthesis at the transcriptional level and depletion of several lipid classes at 3-3.5 months of age, we found accumulation of lipid droplets in astrocytes of TwKO^{astro} mice at later stages. This is at first glance surprising, however shuttling of the lipid molecules to lipid droplets is a protective mechanism against lipotoxicity (Nguyen et al. 2017;

Bailey et al. 2015). Formation of lipid droplets is well documented to increase in various brain pathologies and with ageing, which may constitute an adaptive mechanism (reviewed in (Farmer et al. 2020)). In Drosophila, lipid droplets accumulated in glia in response to neuronal mitochondrial dysfunction, but not upon glial cell-autonomous mitochondrial dysfunction (L. Liu et al. 2015). Our findings suggest that in mice cell-autonomous mitochondrial dysfunction in astrocytes can also promote lipid droplet formation. Lipid droplets also accumulated in astrocytes and microglia of mice with global *Ndufs4* knockout and upon administration of Complex I inhibitor rotenone (L. Liu et al. 2015, 2017). In mice, it was not however established whether the stimulus to generate lipid droplets is cell autonomous.

Lipid import. A recently established astrocyte function is to import and metabolise lipids generated by neurons (Ioannou et al. 2019). These imported lipids are metabolised in astrocytes via mitochondrial **β**-oxidation (Ioannou et al. 2019). While we did not investigate the efficacy of **β**-oxidation in astrocytes upon *Twnk* knockout, highly abnormal mitochondrial morphology suggests that organellar function is impaired. One outcome of this can be disrupted import of **β**-oxidation enzymes and fatty acyls to mitochondria. Accumulation of long-chain carnitines, which require active import to mitochondria to be metabolised through **β**-oxidation, further supports this hypothesis. If the astrocytes in TwKO^{astro} mice continue to import lipids but have impaired **β**-oxidation, it is possible that lipids accumulate in the cytoplasm, triggering signalling to downregulate lipid biosynthesis and to shuttle excess lipids to lipid droplets. Direct investigation of this hypothesis in TwKO^{astro} mice is complicated, as even if **β**-oxidation would be found to be impaired, it would be a challenge to restore this function. If the hypothesis is correct, we however may expect that disruption of **β**-oxidation in astrocytes would result in disturbances of lipid homeostasis similarly to TwKO^{astro} mice.

In striking contrast to TwKO^{astro} mice, lipid composition of TwKO^{neuro} mice remained almost unaffected (see 5.4.2.1). This may suggest the following: i) cell responses of neurons differ compared to astrocytes; ii) neurons contribute less to the homeostasis of the lipid classes that are changed in TwKO^{astro} mice; iii) TwKO^{neuro} tissue is preserved better, as vacuolation occurring in TwKO^{astro} might be reflected in the levels of measured metabolites.

6.3.3 Ciliogenic program

Twnk knockout in astrocytes induced the anomalous expression of transcription factor FOXJ1 and motile cilia components in astrocytes (Figure 39). Astrocytes also displayed contorted cilia morphologies, higher maximum length of cilia, and a shift in length towards longer cilia (Figure 39).

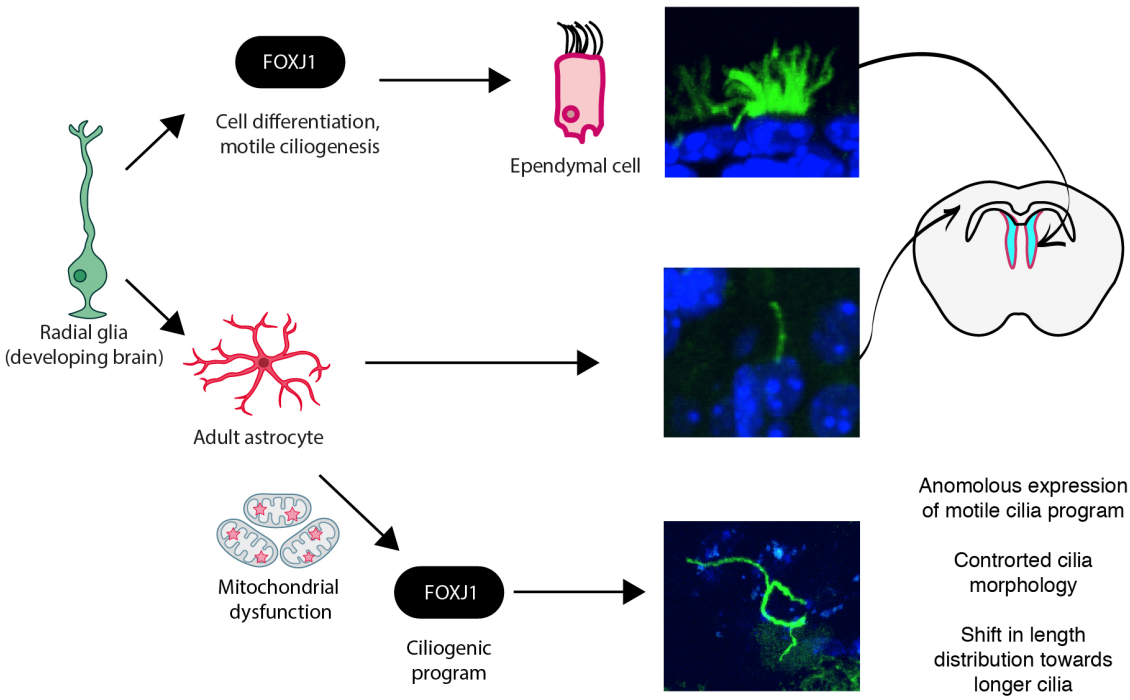

Figure 39. Schematic. Mitochondrial dysfunction induces a ciliogenic program in astrocytes (from (III)).

Cilia are the oldest known organelle, as they were first discovered by Leewenhoek in the 17th century (reviewed in (Beales and Jackson 2012)). Cilia are also ancient organelles, likely present in the last eukaryotic common ancestor (reviewed in (Yubuki and Leander 2013; Hodges et al. 2011)). Motile cilia have a function in cell motility and facilitate liquid movement at the cell surface, while primary cilia have sensory and signalling functions (reviewed in (Nachury and Mick 2019)). The membrane of primary cilia compartmentalises receptors to several signalling pathways, including those indispensable in development (reviewed in (Nachury and Mick 2019)). Additionally, the role of cilia in the cell cycle is apparent (reviewed in (Izawa et al. 2015)). Primary cilium emerges upon the cell cycle exit from a mother centriole. For a cell to re-enter the cell cycle, the cilium needs to resorb and release the centriole pair. The majority of differentiated eukaryotic cells, including astrocytes, possess a primary cilium (Sterpka and Chen 2018; Kasahara et al. 2014; Dahl 1963; Karlsson 1966). The post-developmental functions of primary cilia are less understood, however these organelles can act as a signalling center in response to cell stress (Walz 2017; Louvi and Grove 2011).

Regulation of the motile ciliogenesis program. We discovered that the expression of motile cilia components is regulated upon various stimuli in astrocytes, which are known to possess only an immotile cilium. HBEGF and EGFR inhibition resulted in a robust induction of the motile ciliogenesis program (see 5.5). Stimulation with an inflammatory cocktail or trauma led to downregulation of the program, which however appeared partial (see 5.5). The program was not regulated in aged astrocytes or in mice with heart-specific knockout of *Twnk* and mitochondrial factors. This highlights the specificity of the response, however it remains to be discovered for what range of pathologies this may be relevant. We hypothesize that the activity of FOXJ1 is key in regulating the expression of motile cilia components in astrocytes. FOXJ1 is indispensable for differentiation of multiciliated cells in mammals, and controls the expression of an array of motile cilia components (Jacquet et al. 2009; You et al. 2004; Didon et al. 2013). FOXJ1 acts in coordination with transcription factors of the RFX family, and we also found enrichment of motifs recognised by these factors in promoters of genes upregulated in our dataset (III).

Foxj1 and the ectopic formation of motile cilia. In xenopus and zebrafish, ectopic expression of FOXJ1 during embryogenesis induced the formation of motile cilia in cells, which are normally devoid of these organelles (Xianwen Yu et al. 2008; Stubbs et al. 2008). These cilia were solitary and longer than primary cilia, functionally motile, and had corresponding ultrastructural organisation (Xianwen Yu et al. 2008; Stubbs et al. 2008). Consequences for cell homeostasis of forced induction of motile cilia remain unknown. In mammalian cells, overexpression of FOXJ1 induced cilia elongation, but the functional motility or ultrastructure of these cilia were not investigated (Cruz et al. 2010).

What consequences can arise from induced expression of *Foxj1* and motile cilia components in astrocytes? It is tempting to speculate that our findings are compatible with the formation of a motile monocilium. The important difference to experiments with xenopus and zebrafish is however that when FOXJ1 expression was induced during embryogenesis, ectopic motile monocilia were likely formed *de novo* in non-ciliated cells (Xianwen Yu et al. 2008; Stubbs et al. 2008). In turn, astrocytes of TwKO^{astro} mice at the time of *Foxj1* induction likely possessed a primary cilium. If the expression of motile cilia components is induced at the protein level, it is possible that such factors are trafficked to the primary cilium to remodel its organisation. It is also possible that motile cilia factors are trafficked to the primary cilium, but only form aberrant complexes or aggregations. This might cause the axoneme structure to manifest with observed contorted phenotypes. Another possibility is that the primary cilium is first lost by resorption of shedding (Mirvis et al. 2019). Then, the growth of the motile monocilium could occur *de novo*. Astrocytes of TwKO^{astro}, similarly to other reactive astrocytes, upregulate the expression of several genes and signalling pathways, which are normally active in radial glia but silenced in mature astrocytes (discussed in 2.2.1.2). Whether such partial de-differentiation involves the temporary loss of a primary cilium as is observed before entering a cell cycle remains to be investigated. Finally, aberrant induction of motile cilia components also may result in their degradation or aggregation in the cytoplasm. This can affect cell homeostasis via other mechanisms than ciliary function, or resolve asymptomatically.

The motile ciliogenesis program and multiciliated cell differentiation. In development, the motile ciliogenesis program is tightly connected to differentiation of multiciliated ependymal cells. In astrocytes purified from TwKO^{astro} mice, we found the upregulation of expression of several factors that govern key stages of multiciliated cell differentiation. One of those genes was *Trp73* ,which encodes the tumor suppressor factor p73. P73 governs the

differentiation of multiciliated cells by regulating those key factors in the signalling cascade which were upregulated in our dataset, including FOXJ1 (Fujitani, Sato, and Yamashita 2017; Marshall et al. 2016; Marques et al. 2019; Napoli and Flores 2016; Nemajerova et al. 2016). The role of FOXJ1 in development is to drive differentiation of ependymal cells from radial glia, as FOXJ1 is essential for both cell differentiation and the formation of motile cilia (Vidovic et al. 2018; Jacquet et al. 2009).This might suggest a role for p73 upstream of motile cilia response in astrocytes. Notably, p73 also coordinates cell responses to metabolic challenges by governing adaptive glucose metabolism (Nemajerova et al. 2018; Du et al. 2013). Putatively, this connects metabolic status of astrocytes upon mitochondrial dysfunction to upregulation of motile ciliogenesis program. In summary, the transcriptional program induced in astrocytes upon mitochondrial dysfunction may correspond to developmental program for multiciliated cell differentiation.

Contorted morphology. The morphology of cilia was changed upon astrocytic knockout of *Twnk* both *in vivo* and in cultured cells (see 5.4.3.2). Some cilia presented with extremely contorted morphologies, which were never found in respective controls. The maximum length of cilia was also increased, and there was an overall shift in length distribution towards longer cilia (see 5.4.3.2). Extremely contorted cilia appeared to be much longer than any other cilia, although due to multiple loops the length of such cilia could not be reliably measured, and was not included to the analysis of cilia length distribution. It is possible that contortion occurs as a result of physical constraints to accommodate very long cilia in a dense intracellular environment. It is tempting to speculate that the length of primary cilia might affect signalling functions, for example via changes in distribution of receptors.

Cilia and mitochondrial dysfunction. Ciliary responses were previously linked to mitochondrial dysfunction in dividing cultured cells and in development. Inhibition of OXPHOS resulted in cilia elongation in human neuroblastoma and retinal pigmented epithelial cell lines (Bae et al. 2019). Primary cilia were also elongated in cultured fibroblasts derived from a patient with the mtDNA depletion syndrome (the pathogenic variant in *Mpv17* gene) and from a patient with the impaired mitochondrial iron-sulfur cluster metabolism (Burkhalter et al. 2019). Inhibition of OXPHOS function and knockdown of multiple mitochondrial genes in developing zebrafish led to elongation of embryonic motile cilia (organelles which initiate right-left symmetry positioning) (Burkhalter et al. 2019). This was also associated with abnormal cilia function and resulted in heterotaxy, mimicking human diseases associated with cilia dysfunction (Burkhalter et al. 2019). Finally, a whole-genome RNA interference screen identified that inhibition of mitochondrial gene expression induces ciliogenesis (Failler et al. 2020). We provided evidence that ciliary responses can be activated also in differentiated cells in response to mitochondrial dysfunction. Additionally, we discovered a novel transcriptional regulation of ciliary response. Future research would be needed to elucidate the role of these responses in the pathogenesis of mitochondrial dysfunction.

CONCLUSIONS AND FUTURE PERSPECTIVES

Historically, the research effort to investigate the pathogenesis of mitochondrial dysfunction in the central nervous system was concentrated on neuron-driven consequences. Astrocytes are cells indispensable for brain homeostasis, which were known to respond to mitochondrial dysfunction with upregulation of expression of intermediate filaments. This response was assumed to be secondary to the neuronal pathology, and how astrocyte function and fitness are affected by mitochondrial dysfunction was not known.

The research presented in this thesis challenges the established understanding of the pathogenesis of mitochondrial dysfunction in the central nervous system. Mitochondrial dysfunction in astrocytes alone was sufficient to drive brain pathology and induce cell-autonomous responses. These findings help to understand the importance of mitochondrial function for astrocyte homeostasis and are relevant to consider when investigating mitochondrial encephalopathies and brain diseases with secondary mitochondrial dysfunction. Additionally, we discovered novel astrocytic responses to mitochondrial dysfunction. These include downregulation of lipid biosynthesis and an induction of the ciliogenic program. These findings uncover a new intracellular signalling axis for the central nervous system.

Conditional knockout of *Twnk* or *Cox10* in astrocytes led to vacuolation of brain parenchyma, referred to as spongiotic encephalopathy. The pathology peaks the interest as it is similar to several mitochondrial encephalopathies, however the cellular basis of this pathology in humans remains to be established. No therapies exist to combat this pathology, and we concluded that administration of rapamycin or a ketogenic diet were ineffective treatment strategies. The research using conditional gene knockouts of essential mitochondrial proteins was useful to establish astrocytes as a relevant cell type for the pathogenesis of mitochondrial encephalopathies, however investigation of human diseases would require novel models that faithfully recapitulate both the cause and the manifestation of a disease of interest. Only a limited number of such models are available for rare genetic mitochondrial diseases, presenting a challenge for the field in developing new therapeutics, as well as to carry out appropriate preclinical testing of the existing approaches.

Our findings that mitochondrial dysfunction in astrocytes results in a profound dysregulation of lipid metabolism provide conceptual evidence of the importance of astrocyte function to brain lipid homeostasis. The finding that a ciliogenic program is induced in astrocytes upon mitochondrial dysfunction uncovers a novel signalling link between the two fundamental organelles. It is of great interest to understand how cilia contribute to cell adaptation and pathology upon mitochondrial dysfunction, and to elucidate what consequences can anomalous induction of motile cilia components have for cells that are normally devoid of these organelles.

Finally, our research provides the first comprehensive characterisation of astrocyte transcriptional responses to mitochondrial dysfunction. Together, the research presented in this thesis provides a solid base for future efforts to target cell responses to combat pathologies induced by mitochondrial dysfunction in astrocytes.

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